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PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches in improving quality on issues pertaining to tropical agriculture and its related fields.

Pertanika Journal of Tropical Agricultural Science currently publishes 6 issues per year (*January, February, May, June, August, and November*). It is considered for publication of original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

The journal is available world-wide.

Aims and Scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

History

Pertanika was founded in 1978. Currently, as an interdisciplinary journal of agriculture, the revamped journal, *Pertanika* Journal of Tropical Agricultural Science now focusses on tropical agricultural research and its related fields.

Vision

To publish journals of international repute.

Mission

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 90 days. The elapsed time from submission to publication for the articles averages 180 days. We are working towards decreasing the processing time with the help of our editors and the reviewers.

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Pertanika Journal of Tropical Agricultural Science is now over 47 years old; this accumulated knowledge has resulted in *Pertanika* Journal of Tropical Agricultural Science being abstracted and indexed in Journal Citation Reports (JCR-Clarivate), SCOPUS (Elsevier), BIOSIS, National Agricultural Science (NAL), Google Scholar, MyCite, and ISC.

Citing Journal Articles

The abbreviation for *Pertanika* Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Publication Policy

Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in proceedings.

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The *Pertanika* journals and Universiti Putra Malaysia take seriously the responsibility of all its journal publications to reflect the highest publication ethics. Thus, all journals and journal editors are expected to abide by the journal's codes of ethics. Refer to *Pertanika*'s **Code of Ethics** for full details, available on the official website of *Pertanika*.

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 $\leq 20\%$ – *PASS*; $> 20\%$ – *REJECT*.

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An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media—print and electronic. All *Pertanika* journals have an e-ISSN.

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Lag Time

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For manuscript preparation, authors may refer to *Pertanika*'s **INSTRUCTION TO AUTHORS**, available on the official website of *Pertanika*.

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Authors who complete any submission are notified with an acknowledgement containing a manuscript ID on receipt of a manuscript, and upon the editorial decision regarding publication.

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Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

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In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of the written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and Editor-in-Chief examine the paper to determine whether it is relevance to the journal's needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If it is not appropriate, the manuscript is rejected outright and the author is informed.
2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions to strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors to attend to the reviewers' suggestions and perhaps additional advice on how to revise the manuscript.
4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by the reviewers.
5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.
6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.
7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika*. An acceptance notification is sent to all the authors.

The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted online.

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Foreword

Welcome to the third issue of 2026 of the *Pertanika Journal of Tropical Agricultural Science* (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 28 articles: one short communication; seven review articles; and the rest are regular articles. The authors of these articles come from different countries namely Brunei Darussalam, Central America, China, India, Indonesia, Japan, Korea, Malaysia, Philippines, Saudi Arabia, Taiwan, The Gambia, Thailand, and Vietnam.

The regular article entitled “Influence of Mother Palm Age and Harvesting Maturity Seed Nut on Seed Quality and Germination of Aromatic Pandan Coconut” reports on strategies to optimise the germination performance and seed quality of the Aromatic Green Dwarf (AROD) variety. To address the low germination rates typically observed under commercial practices, the study evaluated the synergistic effects of mother palm age (10 years versus 30 years) and harvesting maturity stages at 10, 11, and 12 pollinations (MAP) for germination. Comprehensive assessments over four months, measuring final germination rate, germination index, mean germination time, germination rate index, time spread, and seedling vigour index, revealed that harvesting at an earlier maturity stage of 10 MAP significantly enhanced seed viability. Specifically, aromatic pandan coconut seed nuts collected at 10 MAP achieved the highest final germination rates for both 10-year (60.7%) and 30-year (65.3%) mother palms, demonstrating that strategic harvest timing can substantially enhance nursery success and support high-quality planting material production. Further details of this study can be found on page 1095.

The article titled “SSR Markers for Verifying Crosses and Assessing Whitefly Resistance in Soybean Breeding Programme” focuses on establishing a robust molecular selection technique to identify true hybrids during the hand-pollination phase of soybean breeding. In this research, the whitefly-susceptible soybean variety Anjasromo was crossed with resistant donor germplasms, IAC 100 and G100H. Whitefly resistance across parental lines and 46 F₁ progenies was evaluated in greenhouse settings using a free-choice host test, while molecular

verification of true crosses was executed using the specific SSR marker Satt045. The findings showed severe leaf damage reaching up to 84.7% in Anjasmoro and unsuccessful crosses, which precisely mirrored the genetic banding pattern of the susceptible parent. Conversely, true hybrid offspring exhibited significantly reduced leaf damage and a combined parental banding pattern, underscoring that combining greenhouse resistance screening with SSR marker analysis offers a highly reliable framework for accelerating whitefly-resistant crop development. Full details of this study are available on page 1157.

The review article titled “Methionine, Lysine and Tryptophan Optimisation in Broiler Nutrition: Functional Roles, Supplementation Strategies and Feed Crop Biofortification” presents an integrated overview of precision nutrition approaches designed to support high-quality lean tissue growth, feed efficiency, and immune performance in sustainable poultry production. The authors examine the critical functional roles of key limiting amino acids, detailing how methionine (Met) drives protein synthesis and antioxidant defences, lysine (Lys) controls muscle and carcass development, and tryptophan (Trp) regulates appetite, stress adaptation, and serotonin synthesis. Beyond short-term precision feed formulations utilising crystalline amino acids to balance low-crude-protein (CP) commercial diets, the paper comprehensively evaluates long-term solutions such as agricultural biofortification, transgenic approaches, and CRISPR/Cas genome editing to intrinsically improve the nutritional profiles of baseline feed crops like maize, soybean, and rice. The synthesis highlights the necessity of multi-omics feeding trials and integrated agro-economic frameworks to successfully adopt these sustainable broiler nutrition strategies globally. Further detailed information can be found on page 1363.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika’s stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers and Editorial Board Members of PJTAS, who have made this issue possible.

PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Editor-in-Chief

Md Kamal Uddin

The Effect of Chitinase Enzyme on Black Soldier Fly Larvae Meal to Optimise Its Use as a Sustainable Protein Source in Ruminants: An In Vitro Study

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ABSTRACT

Chitin, a major component of the insect exoskeleton, serves as a source of dietary fibre but also acts as an anti-nutritional factor, limiting the bioavailability of protein and minerals in black soldier fly (BSF) larvae. Chitinase enzymes, typically secreted by chitinolytic bacteria such as Actinomycetes, can hydrolyse chitin into simpler, digestible forms. This study aimed to evaluate the nutrient profile and in vitro digestibility of BSF larval meal hydrolysed with chitinase enzyme derived from *Streptomyces* BLH 5-14. This study was set up with the following treatments: soybean meal; BSF meal without chitinase (EC0); and BSF larval meal treated with chitinase at levels of 2, 4, and 6

units per 100 grams (EC2, EC4, and EC6), each replicated six times in a randomised complete design. The results indicated that chitinase treatment reduced chitin and acid detergent fibre (ADF) content, while crude protein and neutral detergent fibre (NDF) showed varying trends. ADF content in the EC2 treatment declined from 25.51% to 17.28%, and chitin content dropped from 8.47% to 4.59%. Although bacterial rumen populations rose when the dose was increased to 6 U/100 g, the chitinase enzyme administration up to the level 6 U/100 g to BSF larval meal was insufficient to improve overall digestibility in both the rumen and post-rumen phases.

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Protein digestibility in the rumen and post-rumen was numerically increased by 12.30% and 5.51%, respectively, in comparison to soybean meal. In conclusion, the application of chitinase enzyme up to level 6 U/100 g to BSF larval meal was inadequate to enhance the overall digestibility in both the rumen and post-rumen stages.

Keywords: Black soldier fly meal, chitinase, digestibility, protein, *Streptomyces*

INTRODUCTION

Insect meals have recently gained notoriety as a sustainable source of protein for animal feed (Jayanegara et al. 2017a; Linh et al. 2024; Vargas-Serna et al. 2025; Zou et al. 2024). Supported by its simple production system and fast growth rate, Black Soldier Flies (*Hermetia illucens*) have been especially noted for their capacity to effectively transform low-quality organic material into high-quality nutrients (Liland et al., 2017; Lu et al., 2022; Wang and Shelomi, 2017). Food waste, farm waste, animal manure, and other organic wastes can all be broken down by larvae of this species to provide high-protein biomass (Meneguz et al., 2018; Msangi et al., 2022; Siddiqui et al., 2022). It has been explained that the addition of BSF larval meal to animal feed reduces reliance on unsustainable fishmeal in addition to promoting waste utilisation, and thus supporting circular economy principles (Linh et al., 2024; Vargas-Serna et al., 2025). BSF larval meal thus offers a sustainable and promising substitute for conventional protein sources, with a lower environmental footprint (van Huis, 2022; Veldkamp et al., 2022).

Nutritionally, BSF larval meal contains 45.20-58.10% crude protein (CP), 19-20.70% ether extract (EE), 7.44-9.85% acid detergent fibre (ADF), 12.40-32.70% neutral detergent fibre (NDF), 1.34-3.65% calcium, 0.85-1.11% phosphorus, and 5,325-5,159 kcal kg⁻¹ dry matter (DM) gross energy (Matin et al., 2021). The true protein of BSF is, nevertheless, overestimated due to the nonprotein nitrogen from compounds like chitin, which binds protein. Chitin is present in BSF throughout its life cycle, and its concentrations vary in the different stages: larvae (7.8-9.5%), prepupae (9.1-10.9%), pupae (10.3-10.7%), adult flies (23.7-31.1%), shed exoskeletons (22.4-23.8%), and cocoons (5.6-8.4%) (Soetemans et al., 2020). Chitin, which is a N-acetyl- β -D-glucosamine polymer, provides the insect exoskeleton with its rigidity. However, its high content in BSF larvae meal can limit its nutritional value by reducing *in vitro* digestibility and volatile fatty acid production (Jayanegara et al., 2017a; 2017b). In order to optimise the protein value of BSF larval meal, effective treatments are needed to eliminate the negative effect of chitin as well as to improve digestibility. Arbia et al. (2013) reported that chitin extraction typically involves two key steps, demineralisation and deproteinization, that can be carried out by chemical or biological means. In addition, exogenous protease supplementation has been shown to significantly improve protein digestibility and growth

performance in fish, showing its potential for the optimisation of BSF larval meal as a sustainable protein supplement for animal diets (Haider et al., 2024).

Chitinases have been extensively studied for their ability to hydrolyse colloidal chitin and chitin from black soldier fly larvae into water-soluble N-acetylchitooligosaccharides. Gebele et al. (2024) reported that when 1% endochitinase Chit36-TA from *Trichoderma asperellum* was applied for 24 hours, it led to a hydrolysis degree of 32% for colloidal shrimp chitin and 12% for insect larvae chitin. In addition to its application, chitinase has also been used as a feed additive in growing pigs (Yang et al., 2025) and in *Nile tilapia* (Agbohessou et al., 2024), and it has been shown to positively affect feed efficiency, protein digestibility, and increase beneficial bacteria in the intestines. Moreover, the addition of persimmon peel, which had strong chitinase activity (approximately 50% activity at pH 3.0 and pH 8.0 conditions) to layer diets improved overall *in vitro* digestibility, including protein digestibility, at levels less than 6% (Sangkaew & Koh, 2021). However, previous studies primarily focused on its direct supplementation in monogastric and aquaculture. To the best of our knowledge, this is the first study to conduct the use of chitinase from *Streptomyces* BLH 5-14 to hydrolyse chitin of black soldier flies (BSF) and assess its digestibility in a ruminal *in vitro* assay. We hypothesised that the chitinase enzyme would improve the availability of nutrients, especially crude protein, and subsequently increase ruminal digestibility. Thus, this study aimed to evaluate the nutrient profile and *in vitro* digestibility of BSF larval meal that was hydrolysed with a chitinase derived from *Streptomyces*.

MATERIAL AND METHODS

Ethical Approval

This study has obtained ethical clearance approval in the chemical field from the National Research and Innovation Agency (Approval Number: 006/KE.04/SK/12/2022).

Feedstuffs and Experimental Design

The black soldier fly larval meal and soybean meal are obtained from a poultry shop located in Bandung, West Java, Indonesia. These feedstuffs were ground to pass through a 1.0 mm aperture for further processing. The nutrient composition of these feedstuffs used in this *in vitro* study is presented in Table 1.

The experiment was based on a completely randomised design using five treatments with six replications for each treatment. The treatments consisted of

- SBM : soybean meal
- EC0 : BSF larval meal (without chitinase)

- EC2 : BSF larval meal incubated with chitinase at levels of 2 U/100 g
EC4 : BSF larval meal incubated with chitinase at levels of 4 U/100 g
EC6 : BSF larval meal incubated with chitinase at levels of 6 U/100 g

Table 1
Nutrient content of soybean meal and black soldier fly (BSF) larval meal

Nutrient Contents (% DM)	Feedstuff	
	Soybean Meal	BSF-Larval Meal
Dry Matter	91.07	96.66
Organic Matter	86.77	85.31
Ash	13.23	14.69
Crude Protein	28.39	33.16
Ether Extract	3.58	29.15
Crude Fibre	13.82	12.15
Nitrogen Free Extract (NFE) ^a	40.97	10.85
Total Digestibility Nutrient (TDN)	66.75	84.09
Neutral Detergent Fibre (NDF)	43.41	33.45
Acid Detergent Fibre (ADF)	13.24	25.51
Hemicellulose	30.17	7.93

Note. ^aNFE = 100 – (Ash % + Crude protein % + Crude fat % + Crude fibre %)

Production of Chitinase

The isolate used to produce chitinase is *Streptomyces* BLH 5-14 from the Indonesian cultured collection (InaCC), obtained from the Sulawesi marines, Indonesia. The pre-cultured isolate of *Streptomyces* BLH 5-14 is prepared in a baffled Erlenmeyer flask with a spiral, consisting of 0.4% yeast extract, 1% malt extract, 0.4% glucose (ISP-2) medium, and supplemented with 3% artificial sea water (ASW). The medium is shaken in an incubator shaker for three days at 28°C and 190 rpm.

Chitinase was then produced using a medium composition derived from Narayana & Vijayalakshmi (2009), which is composed of chitin-yeast extract-salts (CYS) medium (yeast extract, 0.5%; MgSO₄·7H₂O, 1.0%; K₂HPO₄, 2.0%); FeSO₄·7H₂O, 0.1%; and colloidal chitin, 5.0%; supplemented with 3% artificial sea water (ASW). The final pH of the medium was adjusted to 7. Colloidal chitin preparation used the method by Sandhya et al. (2004). Production was carried out for seven days in an incubator shaker at 28 °C and 190 rpm. The enzyme was harvested using a centrifuge at 12,000 rpm and 4 °C for 20 minutes, then stored in a refrigerator at 4 °C.

Chitinase Activity Assay

Chitinase activity was measured as described by Akeed et al. (2020). Each reaction contained 0.4 mL of the enzyme solution (diluted 5 times with acetate buffer at pH 5) and 0.5 mL of 1 % (w/v) colloidal chitin in 0.1 M sodium acetate buffer (pH 5.0). Subsequently, the mixture was incubated at 60 °C for 30 minutes, and then the reaction was terminated by 1mL DNS (NaOH 10 g/L, dinitrosalicylic acid $C_7H_4N_2O_7$ 10 g/L, phenol C_6H_6O 2 g/L, Na_2SO_3 0.05g/100mL and sodium potassium tartrate $C_4H_4KNaO_6 \cdot 4H_2O$ 20 g/100mL when using). Each mixture was immediately incubated at 100 °C for 10 min, cooled in an ice bath, and centrifuged at 8,000×g for 5 min at 4°C. The supernatant is measured using a visible spectrophotometer at a wavelength of 540 nm. A standard curve was plotted using N-acetyl glucosamine (NAG, Sigma). One unit of chitinase activity was defined as the amount of enzyme that produced 1 μ mol of GlcNAc per min under reaction conditions.

Incubation of BSF Larval Meal with Chitinase

The hydrolysis method was carried out following Wang et al. (2001), who conducted the enzyme xylanase on the feed, with a slight modification in the hydrolysis temperature. The main enzyme activity detected was chitinase (0.1 units/ml). The BSF larval meal was sprayed using a single-nozzle spray bottle. A total of 100 grams of BSF larval meal was sprayed with a medium solution of enzymes, with volumes of 20, 60, and 60 ml for treatments of 2, 4, and 6 U/100 g, respectively. Subsequently, 40 ml of distilled water was added to the BSF larval meal treated with 2 U/100 grams, and 20 ml of distilled water was added to the BSF larval meal treated with 4 U/100 grams. The treated feeds were then incubated at 29°C for 24 hours, followed by drying for 2 days at 60°C. Prior to being used in an *in vitro* study, the treated feeds were kept in the freezer.

In vitro Procedure

The *in vitro* fermentation was conducted using a modified version of the technique by Goering and van Soest (1970). A 0.5 g sample, 40 mL of McDougall buffer, and 10 mL of cattle rumen fluid were added to an anaerobic bottle (100 mL). The bottles were placed in a shaker water bath and incubated for 24 hours at 39 °C. After 24 hours, the pH was measured, the supernatant was collected, and the residue was dried at 60°C for 48 hours. The residue was collected to analyse nitrogen. The supernatant was analysed for NH_3 (Conway & O'Malley, 1942) and the total population of bacteria and protozoa. For determining *in vitro* pepsin digestibility, a modified version of the procedure by Palmer and Jones (2000) was followed. After 24 hours of anaerobic incubation, the residues in the bottles were washed with distilled water on a vortex mixer, then centrifuged at 2,500 rpm for 10 minutes. The supernatant was discarded, and the procedure was repeated before adding 40 ml of acid

pepsin, which was prepared by dissolving 2 g of pepsin (1:10,000) in 1 L of 0.1 M HCl. Each tube was then thoroughly vortexed and incubated at 39 °C for 24 hours. The tubes were centrifuged, the remaining supernatant was discarded, and the residues were dried at 60 °C for 48 h. Residues were analysed for nitrogen.

Chemical Analyses

AOAC (2002) procedures were carried out to analyse dry matter, ash, ether extract, crude protein, and crude fibre of feed samples, while the determination of neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents followed the methods described by van Soest et al. (1991). Chitin content was determined using the procedure by Black and Schwartz (Black & Schwartz, 1950; Borić et al., 2020). Total digestible nutrient (TDN) content was estimated in accordance with Hartadi et al. (1980).

Statistical Analysis

Data were analysed by ANOVA, and differences were declared significant at $p < 0.05$, tested by Duncan's Multiple Range test, with SAS software version 9.0. Correlation and principal component analyses among parameters were conducted using RStudio software (version 2024, Post Software, PBC).

RESULTS AND DISCUSSION

Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and chitin content of BSF larval meal hydrolysed using different levels of chitinase enzyme are presented in Table 2. Protein content of the non-treated BSF larval meal of the present study was 33.16%, which was similar to the results of Renna et al. (2022) and Pedrazzani et al. (2024). However, the protein content observed was less than in other studies, in which the protein had a value greater than 44% (Jayanegara et al. 2017a; Matin et al. 2021). Moreover, the chitin content was 8.47%, as reported in Soetemans et al. (2020) and Renna et al. (2022). Hydrolysis of BSF larval meal by chitinase 2 U/100 g in this study was the optimal treatment to alter its nutrient content, particularly by reducing ADF and chitin content ($p < 0.0001$). ADF content decreased from 25.51% to 17.28%, and chitin content decreased from 8.47% to 4.59% at this level of enzyme. However, the breakdown of ADF and chitin was not as effectively enhanced by raising the enzyme concentration to 4 and 6 U/100 g. Instead, the crude protein content reduced slightly at these concentrations, showing that chitinase degrades structural carbohydrates and chitin effectively at a level of optimal enzyme level, while excessive application of enzymes could not provide any further advantage.

These findings suggest that chitinase treatment can modify the nutritional profile of BSF larval meal, specifically by reducing chitin and fibre content while potentially enhancing

crude protein to a certain extent. The incomplete hydrolysis of chitin could be attributed to the complex matrix of proteins, chitin, and minerals in BSF larval meal, which may hinder the enzyme's access to chitin. Furthermore, it seems that a one-stage biological procedure, such as hydrolysis with enzymes (Pedrazzani et al., 2024) or fermentation with chitinolytic bacteria (Mulyono et al., 2019), was not sufficient to fully break down chitin complexes. A multi-stage approach combining enzymatic hydrolysis with other methods may be required for more complete chitin breakdown. As reported by Pedrazzani et al. (2024), there is an enhanced ability to remove proteins directly from biomass for the extraction of chitin using enzymatic, chemical, and mechanochemical milling, ultrasonication methods. Pedrazzani et al. (2024) demonstrated that the total protein retained in the BSF larval chitin extract using enzymatic, chemical-enzymatic, and mechanochemical milling, ultrasonication methods was 46.7%, 15.5%, 15.7%, and 13.0%, respectively.

The changes in fermentative characteristics in the rumen of BSF larval meal with the addition of chitinase are presented in Table 3. Significant differences in rumen digestibility of dry matter, total gas production, and total populations of bacteria and protozoa ($p<0.01$), and rumen digestibility of protein ($p<0.05$) were observed among the treatments. Our study has shown that the use of 6 U/100 g of chitinase (EC6) was insufficient to enhance overall digestibility in both the rumen and post-rumen phases, despite the fact that bacterial rumen populations increased when the dose was increased to 6 U/100 g. Digestibility of protein in the rumen and post-rumen for the BSF larval meal treatment at 6 U/100 g chitinase (EC6) was higher by 12.30% and 5.51%, respectively, compared to soybean meal. This suggests that although chitinase hydrolysed the nutrient profile of the BSF larval meal, digestibility was not always improved beyond a certain point. This could be due to a number of factors, such as enzyme saturation, the structural resistance of the remaining chitin and fibre, or possible interactions between the hydrolysed components and microbial digestion.

Table 2
Nutrient content of BSF larval meal incubated with chitinase enzyme

Nutrient Contents (% DM)	Incubate BSF-larval Meal with Chitinase Enzyme (U/100 g)				SEM	p-value
	EC0	EC2	EC4	EC6		
Crude Protein	33.17b	34.05a	32.36d	32.62c	0.20	<.0001
Neutral Detergent Fibre (NDF)	33.45	29.71	35.83	29.27	1.26	0.2021
Acid Detergent Fibre (ADF)	25.51a	17.28d	21.92b	19.63c	0.94	<.0001
Hemicellulose	7.93	12.43	13.91	9.64	1.14	0.2565
Chitin	8.47a	4.59b	5.04b	7.65a	0.51	<.0001

Note. Means in the same row with different letters differ significantly; SEM: standard error of the means; EC0 - BSF larval meal; EC2 - BSF larval meal incubated with chitinase enzyme (2 U/100 g); EC4 - BSF larval meal incubated with chitinase enzyme (4 U/100 g); EC6 - BSF larval meal incubated with chitinase enzyme (6 U/100 g)

Table 3
In vitro rumen fermentation and post-ruminal digestibility of soybean meal and hydrolysed BSF larval meal

Parameters	Treatments					SEM	p-value
	SBM	EC0	EC2	EC4	EC6		
Rumen Digestibility 24h							
Dry Matter (IVDMD24), %	56.97a	42.85b	47.19b	33.48c	47.52b	1.92	0.0005
Protein (IVCPD24), %	49.83ab	54.70a	58.69a	41.54ab	55.96a	1.77	0.0105
Pepsin Digestibility							
Dry matter (IVDMD48), %	71.74	70.17	68.78	68.76	72.71	1.72	0.94
Protein (IVCPD48), %	77.67	77.78	78.72	75.73	81.95	0.85	0.2202
NH ₃ (mM)	19.20	31.67	24.00	28.40	25.50	3.12	0.7676
pH	7.00	6.92	6.92	7.00	7.00	0.02	0.56
Gas Production 24h, mL/200 mg DM	32.53a	11.49bc	10.27c	12.13b	10.88bc	1.14	<.0001
a	0.31	0.02	0.14	0.00	0.00	0.06	0.382
b	33.14a	15.57b	13.98c	14.79bc	14.53bc	1.04	<.0001
c	0.14a	0.07b	0.08b	0.09b	0.07b	0.01	<.0001
a + b	33.45a	15.59b	14.12c	14.79bc	14.53bc	1.05	<.0001
Total Population of Bacteria (×10 ⁹ cfu/mL)	3.54b	2.34c	2.55c	3.37b	4.30a	0.15	<.0001
Total Population of Protozoa (×10 ⁶ cells/mL)	1.57e	8.95a	6.21b	4.61c	3.43d	0.48	<.0001

Note. Means in the same row with different letters differ significantly; SEM: standard error of the means. a - initial gas production (mL/200 mg DM), b - gas production during incubation (mL/200 mg DM); a + b - potential gas production (mL/200 mg DM); c - fractional rate of gas production per hour; SBM: Soybean meal; EC0 - BSF larval meal; EC2 - BSF larval meal incubated with chitinase enzyme (2 U/100 g); EC4 - BSF larval meal incubated with chitinase enzyme (4 U/100 g); EC6 - BSF larval meal incubated with chitinase enzyme (6 U/100 g)

On the other hand, BSF meal treatment had a lower rate of DM digestibility in the rumen than soybean meal (SBM). The lower dry matter digestibility of BSF larval meal might be attributed to its high ether extract content (approximately 29%), which is known to exert a toxic effect on cellulolytic bacteria, thereby reducing fibre digestibility (Behan et al., 2019; Lima et al., 2017). Additionally, the presence of chitin, which was not optimally hydrolysed by chitinase, may have further contributed to the reduced digestibility. Previous studies evaluating the addition of BSF larval meal instead of SBM to ruminant diets have shown that BSF larval meal generally has lower nutritional value due to reductions in IVDMD caused by their chitin and high fat content (Jayanegara et al. 2017b). However, inclusion of defatted BSF larval meal substitution up to 40% of SBM (6.4% in TMR) exhibited higher IVDMD and IVNDFD levels (Kahraman et al., 2023). Notably, in this study, the post-rumen digestibility of BSF larval meal treated with chitinase was similar to that of SBM.

In the present study, ruminal ammonia (NH_3) concentrations in vitro ranged from 19.20 to 31.67 mM, representing a state that is conducive to the development of robust rumen microbes. This range is broader than the 5 to 11 mM range established by Schwab and Broderick (2017) as being most optimal to sustaining microbial nitrogen flow at its optimal level, whose optimal ammonia content is determined by diet and fermentative dynamics. The increased NH_3 concentration in the presence of added black soldier fly meal might be attributed to the increased degradability of its crude protein in the rumen. The result coincided with that obtained by Renna et al. (2023), where BSF larval meal (2.49 mmol/g DM) showed higher ammonia production than SBM and rapeseed meal, 4.48 and 3.06 mmol/g DM, respectively. In contrast to previous research, wherein it was implied that the incorporation of 40% (Kahraman et al., 2023) and 50% (Jayanegara et al., 2017b) BSF larval meal into total mixed rations led to the reduction in NH_3 -N levels, in the current study, no significant difference ($p > 0.05$) was observed. The difference is due to particular protein fractions present in BSF larval meal, which could have varied degradation kinetics compared to soybean meal. Kahraman et al. (2023) explained that soybean meal's high proportion of rapidly (B1) and intermediately (B2) degradable protein fractions may be behind its impact on NH_3 -N concentration.

Furthermore, ruminal pH value in the present work was 6.92 to 7.08, which falls within the ideal range of 6.8 to 7.2 for facilitating rumen digestion (Phesatcha et al., 2021; Totakul et al., 2021). The stability of ruminal pH suggests that the addition of the chitinase enzyme did not exert a significant effect ($p < 0.05$) on this parameter, thereby maintaining an environment conducive to optimum microbial activity. In line with this, the results suggest that the addition of chitinase tends to alter the rumen microbial population. The use of 6 U/100 g chitinase (EC6) resulted in an increase in the total bacterial population from 2.34×10^9 cfu mL^{-1} to 4.30×10^9 cfu mL^{-1} , and a decrease in the protozoan population from 8.95×10^6 cells mL^{-1} to 3.43×10^6 cells mL^{-1} .

Interestingly, in the current study, the treatment of BSF larval meal with chitinase did not increase the total gas production (Table 3, Figure 1). The lower gas production in BSF larval meal (19.25-20.21 mL/200 mg DM), even with chitinase treatment, may be attributed to the composition of the substrate. In comparison, soybean meal, characterised by a higher fermentable fraction, demonstrated significantly greater gas production (31.26 mL/200 mg DM). Treatment SBM showed the highest values for both b (33.14 mL) and c (0.14 mL/h), indicating more extensive and rapid fermentation. Meanwhile, treatments with BSF larval meal (EC0-EC6) produced significantly less gas from the insoluble fraction, with b values ranging from 13.98 to 15.57 mL, and showed lower fermentation rates ($c = 0.07$ -0.09 mL/h). Protein and fat, which are high in BSF larval meal, contribute little to gas and VFA production (Getachew et al., 1998).

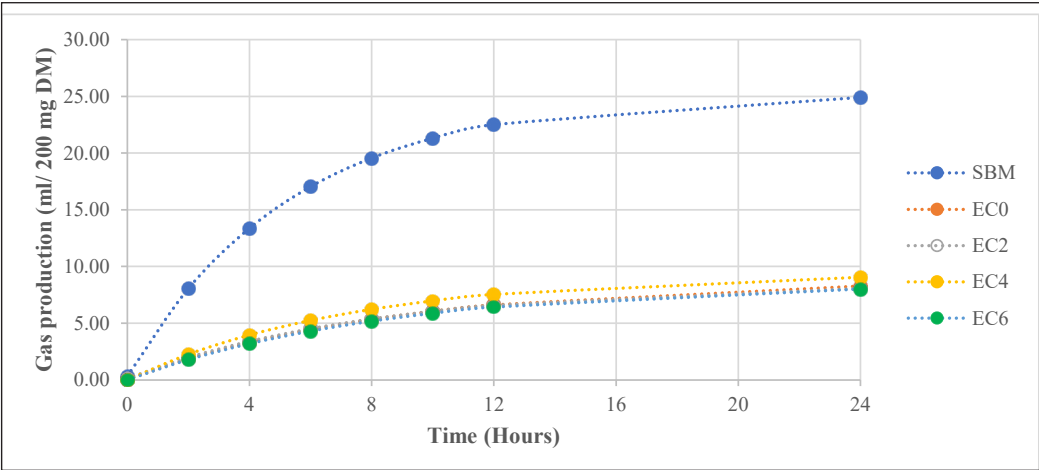


Figure 1. Cumulative total gas production of experimental diets during 24h incubation

Even the different sources of nitrogen, including NPN at similar concentrations of total ammonia nitrogen (0 - 128 mmol/L), as reported by Shen et al. (2023), could influence microbial populations and *in vitro* rumen fermentation profiles (gas production, dry matter digestibility, total volatile fatty acid, acetate, propionate). BSF larval meal, despite being treated with chitinase, has a higher fibre and ether extract (EE) content, which is less fermentable, thus explaining the lower gas production (Jayanegara et al. 2017a). The authors explained that BSF larval meals had lower total gas production at 24 hours than SBM, with recorded values of 214.5 and 93.8-115.8 mL g⁻¹ DM, respectively, which is due to their higher fibre and EE content (Jayanegara et al., 2017a). This finding suggests that while chitinase may alter microbial populations, the fermentation indices and gas production are strongly influenced by the composition of the substrates, particularly their fermentable carbohydrate content and fibre levels.

Treatment EC6 was also reported to possess the ability to enhance fermentation efficiency and microbial balance, indicating a change in the nutritional profile of BSF larval meal. As illustrated in Figure 2, chitin was positively related to ADF and negatively to hemicellulose, which means that the higher the ADF content, the lower the digestibility and nutrient availability can be (Nurdianti et al., 2024; Sándor et al., 2022). Moreover, near interrelations were established among digestibility coefficients and parameters of fermentation, GP24 having positive correlations with IVDMD24 and negative with protozoa, while IVDMD values were highly correlated with N-NH₃ and IVCPD. These correlations were also supported by PCA analysis (Figure 3), which showed that treatment EC6, as well as SBM, sustained better fermentation and a favourable microbial population. On the other hand, treatments EC0, EC2, and EC4 were associated with greater protozoa and ammonia-N content, suggesting reduced nitrogen utilisation and fermentation indices.

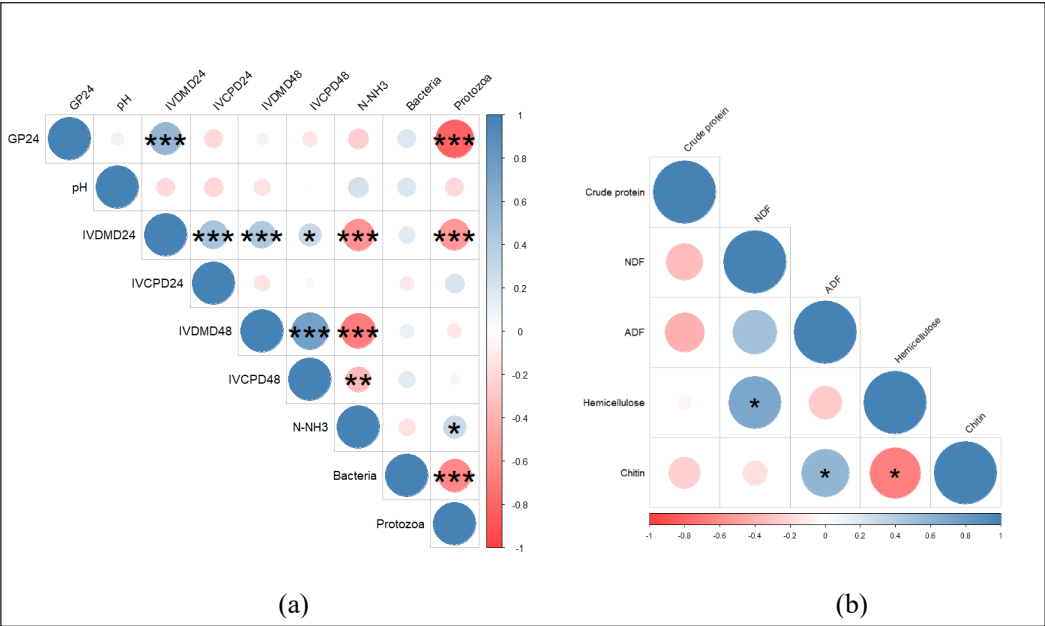


Figure 2. Correlation plot between (a) in vitro digestion and fermentation parameters (n = 60) and (b) chemical composition (n = 12). Positive and negative correlation coefficients are represented by blue and red colour scales, respectively. Significance levels: ***p < 0.001, **p < 0.01, *p < 0.05

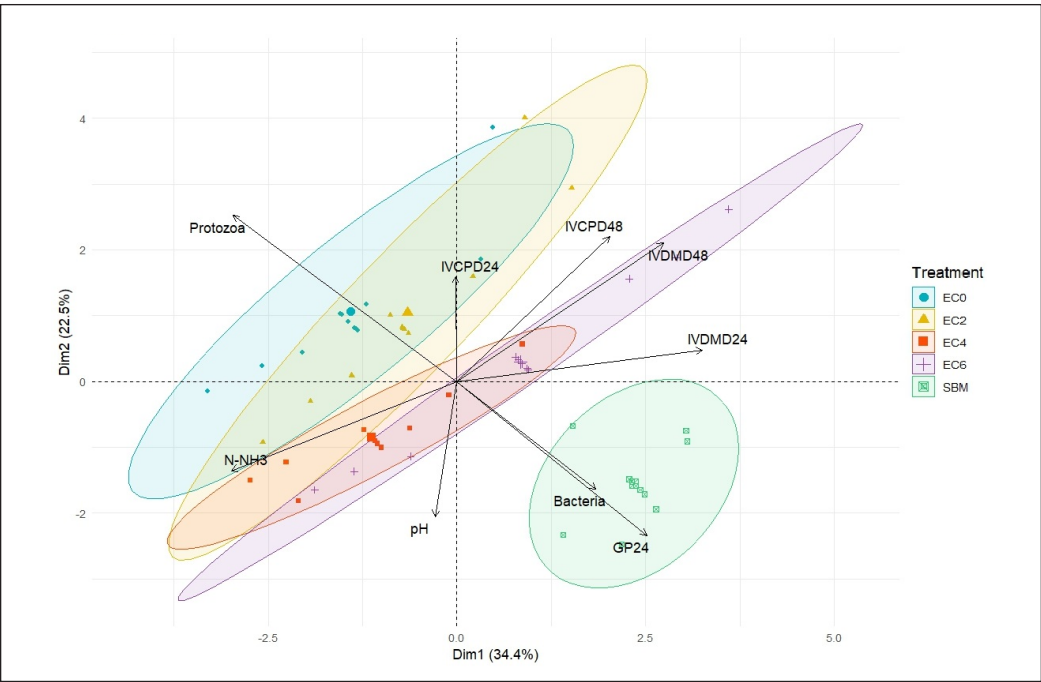


Figure 3. PCA of soybean meal and hydrolysed BSF larval meal

CONCLUSION

The application of chitinase enzyme up to level 6 U/100 g to BSF larval meal was insufficient to improve the overall *in vitro* ruminal and post-ruminal digestibility. However, chitin and acid detergent fibre contents were considerably decreased by treatment with 2 U/100 g, while bacterial rumen populations increased when the dose was raised to 6 U/100 g. Further research is needed to determine the optimal dosage of enzymes and complementary methods to optimise the digestibility and fermentation characteristics of BSF larval meal.

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REFERENCES

- Agbohessou, P. S., Mandiki, R., Mes, W., Blanquer, A., Gérardy, M., Garigliany, M. M., Lambert, J., Cambier, P., Tokpon, N., Lalèyè, P. A., & Kestemont, P. (2024). Effect of fatty acid-enriched black soldier fly larvae meal combined with chitinase on the metabolic processes of Nile tilapia. *British Journal of Nutrition*, 131(8), 1326-1341. <https://doi.org/10.1017/S0007114523003008>
- Akeed, Y., Atrash, F., & Naffaa, W. (2020). Partial purification and characterisation of chitinase produced by *Bacillus licheniformis* B307. *Heliyon*, 6(5), Article e03858. <https://doi.org/10.1016/j.heliyon.2020.e03858>
- Arbia, W., Arbia, L., Adour, L., & Amrane, A. (2013). Chitin extraction from crustacean shells using biological methods: A review. *Food Technology and Biotechnology*, 51(1), 12-25.
- Association of Official Analytical Chemists. (2002). *Official methods of analysis* (17th ed., Vol. 1, pp. 120-155). AOAC International.
- Behan, A. A., Loh, T. C., Fakurazi, S., Kaka, U., Kaka, A., & Samsudin, A. A. (2019). Effects of supplementation of rumen protected fats on rumen ecology and digestibility of nutrients in sheep. *Animals*, 9(7), Article 400. <https://doi.org/10.3390/ani9070400>
- Black, M. M., & Schwartz, H. M. (1950). The estimation of chitin and chitin nitrogen in crawfish waste and derived products. *Analyst*, 75(889), 185-189. <https://doi.org/10.1039/an9507500185>
- Borić, M., Vicente, F. A., Jurković, D. L., Novak, U., & Likožar, B. (2020). Chitin isolation from crustacean waste using a hybrid demineralisation/DBD plasma process. *Carbohydrate Polymers*, 246, Article 116648. <https://doi.org/10.1016/j.carbpol.2020.116648>
- Conway, E. J., & O'Malley, E. (1942). Microdiffusion methods: Ammonia and urea using buffered absorbents (revised methods for ranges greater than 10 µg. N). *Biochemical Journal*, 36(7-9), 655-661. <https://doi.org/10.1042/bj0360655>

- Gebele, L., Wilke, A., Salliou, A., Schneider, L., Heid, D., Stadelmann, T., Henninger, C., Ahmed, U., Broszat, M., Müller, P., Dusel, G., Krzyżaniak, M., Ochsenreither, K., & Eisele, T. (2024). Recombinant expression and characterisation of the endochitinase Chit36-TA from *Trichoderma asperellum* in *Komagataella phaffii* for chitin degradation of black soldier fly exuviae. *Bioprocess and Biosystems Engineering*, 47(10), 1751-1766. <https://doi.org/10.1007/s00449-024-03067-4>
- Getachew, G., Blümmel, M., Makkar, H. P. S., & Becker, K. (1998). In vitro gas measuring techniques for assessment of nutritional quality of feeds: A review. *Animal Feed Science and Technology*, 72(3-4), 261-281. [https://doi.org/10.1016/S0377-8401\(97\)00189-2](https://doi.org/10.1016/S0377-8401(97)00189-2)
- Goering, H. K., & van Soest, P. J. (1970). *Forage fibre analyses (apparatus, reagent, procedures and some applications)* (Agriculture Handbook No. 379). United States Department of Agriculture.
- Haider, R., Khan, N., Aihetasham, A., Shakir, H. A., Fatima, M., Tanveer, A., Bano, S., Ali, W., Tahir, M., Asghar, M., Farooq, A., Aftab, S., Haq, A. U., & Sarwar, M. (2024). Dietary inclusion of black soldier fly (*Hermetia illucens*) larvae meal, with exogenous protease supplementation, in practical diets for striped catfish (*Pangasius hypophthalmus*, Sauvage 1878). *PLoS ONE*, 19(12), Article e0313960. <https://doi.org/10.1371/journal.pone.0313960>
- Hartadi, H., Reksohadiprodjo, S., Lebdosukojo, S., Tilman, A. D., Kearl, L. C., & Harris, L. E. (1980). *Tables of feed composition for Indonesia*. International Feedstuffs Institute, Utah Agricultural Experiment Station, Utah State University.
- Jayanegara, A., Yantina, N., Novandri, B., Laconi, E. B., Nahrowi, N., & Ridla, M. (2017a). Evaluation of some insects as potential feed ingredients for ruminants: Chemical composition, in vitro rumen fermentation and methane emissions. *Journal of the Indonesian Tropical Animal Agriculture*, 42(4), 247-254. <https://doi.org/10.14710/jitaa.42.4.247-254>
- Jayanegara, A., Novandri, B., Yantina, N., & Ridla, M. (2017b). Use of black soldier fly larvae (*Hermetia illucens*) to substitute soybean meal in ruminant diet: An in vitro rumen fermentation study. *Veterinary World*, 10(12), 1436-1446. <https://doi.org/10.14202/vetworld.2017.1439-1446>
- Kahraman, O., Gülşen, N., İnal, F., Alataş, M. S., İnanç, Z. S., Ahmed, İ., Şişman, D., & Küçük, A. E. (2023). Comparative analysis of in vitro fermentation parameters in total mixed rations of dairy cows with varied levels of defatted black soldier fly larvae (*Hermetia illucens*) as a substitute for soybean meal. *Fermentation*, 9(7), Article 652. <https://doi.org/10.3390/fermentation9070652>
- Liland, N. S., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C. G., Waagbø, R., Torstensen, B. E., & Lock, E.-J. (2017). Modulation of nutrient composition of black soldier fly (*Hermetia illucens*) larvae by feeding seaweed-enriched media. *PLoS One*, 12(8), Article e0183188. <https://doi.org/10.1371/journal.pone.0183188>
- Lima, E. D. S., Valente, T. N. P., Roca, R. D. O., Cezario, A. S., Santos, W. B. R dos, Deminiciis, B. B., & Ribeiro, J. C. (2017). Effect of whole cottonseed or protected fat dietary additives on carcass characteristics and meat quality of beef cattle: A review. *Journal of Agricultural Science*, 9(5), 175-183. <https://doi.org/10.5539/jas.v9n5p175>

- Linh, N. V., Wannavijit, S., Tayyamat, K., Dinh-Hung, N., Nititanarapee, T., Sumon, M. A. A., Srinual, O., Permpoonpattana, P., Doan, H., & Brown, C. L. (2024). Black soldier fly (*Hermetia illucens*) larvae meal: A sustainable alternative to fish meal proven to promote growth and immunity in koi carp (*Cyprinus carpio* var. *koi*). *Fishes*, 9(2), Article 53. <https://doi.org/10.3390/fishes9020053>
- Lu, S., Taethaisong, N., Meethip, W., Surakhunthod, J., Sinpru, B., Sroichak, T., Archa, P., Thongpea, S., Paengkoum, S., Purba, R. A. P., & Paengkoum, P. (2022). Nutritional composition of black soldier fly larvae (*Hermetia illucens* L.) and its potential uses as alternative protein sources in animal diets: A review. *Insects*, 13(9), Article 831. <https://doi.org/10.3390/insects13090831>
- Matin, N., Utterback, P., & Parsons, C. M. (2021). True metabolizable energy and amino acid digestibility in black soldier fly larvae meals, cricket meal, and mealworms using a precision-fed rooster assay. *Poultry Science*, 100(7), Article 101146. <https://doi.org/10.1016/j.psj.2021.101146>
- Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M., & Gasco, L. (2018). Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. *Journal of the Science of Food and Agriculture*, 98(15), 5776-5784. <https://doi.org/10.1002/jsfa.9127>
- Msangi, J. W., Mweresa, C. K., & Ndong'a, M. F. O. (2022). Using organic wastes as feed substrate for black soldier fly larvae. *Journal of Insects as Food and Feed*, 8(4), 441-451. <https://doi.org/10.3920/JIFF2021.0047>
- Mulyono, M., Yunianto, V. D., Suthama, N., & Sunarti, D. (2019). The effect of fermentation time and *Trichoderma* levels on digestibility and chemical components of black soldier fly (*Hermetia illucens*) larvae. *Livestock Research for Rural Development*, 31(10), Article 150. <https://www.lrrd.org/lrrd31/10/mulyo31150.html>
- Narayana, K. J. P., & Vijayalakshmi, M. (2009). Chitinase production by *Streptomyces* sp. Anu 6277. *Brazilian Journal of Microbiology*, 40(4), 725-733. <https://doi.org/10.1590/S1517-83822009000400002>
- Nurdianti, R. R., Dickhoefer, U., & Castro-Montoya, J. M. (2024). Relationship between nutritional composition and fibre digestibility in tropical forages compared to temperate forages. *Italian Journal of Animal Science*, 23(1), 1839-1853. <https://doi.org/10.1080/1828051X.2024.2434697>
- Palmer, B., & Jones, R. J. (2000). The effect of PEG addition in vitro on dry matter and nitrogen digestibility of *Calliandra calothyrsus* and *Leucaena leucocephala* leaf. *Animal Feed Science and Technology*, 85(3-4), 259-268. [https://doi.org/10.1016/S0377-8401\(00\)00125-5](https://doi.org/10.1016/S0377-8401(00)00125-5)
- Pedrazzani, C., Righi, L., Vescovi, F., Maistrello, L., & Caligiani, A. (2024). Black soldier fly as a new chitin source: Extraction, purification and molecular/structural characterisation. *LWT - Food Science and Technology*, 191, Article 115618. <https://doi.org/10.1016/j.lwt.2023.115618>
- Phesatcha, B., Phesatcha, K., Viennaxay, B., Thao, N. T., & Wanapat, M. (2021). Feed intake and nutrient digestibility, rumen fermentation profiles, milk yield and compositions of lactating dairy cows supplemented by *Flemingia macrophylla* pellet. *Tropical Animal Science Journal*, 44(3), 288-296. <https://doi.org/10.5398/tasj.2021.44.3.288>

- Renna, M., Coppa, M., Lussiana, C., le Morvan, A., Gasco, L., & Maxin, G. (2022). Full-fat insect meals in ruminant nutrition: In vitro rumen fermentation characteristics and lipid biohydrogenation. *Journal of Animal Science and Biotechnology*, 13, Article 138. <https://doi.org/10.1186/s40104-022-00792-2>
- Sandhya, C., Krishna, L. A., Nampoothri, K. M., Binod, P., Szakacs, G., & Pandey, A. (2004). Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation. *Journal of Basic Microbiology*, 44(1), 49-58.
- Sándor, Z. J., Banjac, V., Vidosavljević, S., Káldy, J., Egessa, R., Lengyel-Kónya, É., Tömösközi-Farkas, R., Zsolt, Z., Adányi, N., Libisch, B., & Biró, J. (2022). Apparent digestibility coefficients of black soldier fly (*Hermetia illucens*), yellow mealworm (*Tenebrio molitor*), and blue bottle fly (*Calliphora vicina*) insects for juvenile African catfish hybrids (*Clarias gariepinus* × *Heterobranchus longifilis*). *Aquaculture Nutrition*, 2022, Article 4717014. <https://doi.org/10.1155/2022/4717014>
- Sangkaew, M., & Koh, K. (2021). Improvement in the in vitro digestibility of shrimp meal by the addition of persimmon peel. *The Journal of Poultry Science*, 58(1), 51-57. <https://doi.org/10.2141/jpsa.0200024>
- Schwab, C. G., & Broderick, G. A. (2017). A 100-year review: Protein and amino acid nutrition in dairy cows. *Journal of Dairy Science*, 100(12), 10094-10112. <https://doi.org/10.3168/jds.2017-13320>
- Shen, J., Zheng, W., Xu, Y., & Yu, Z. (2023). The inhibition of high ammonia to in vitro rumen fermentation is pH dependent. *Frontiers in Veterinary Science*, 10, Article 1163021. <https://doi.org/10.3389/fvets.2023.1163021>
- Siddiqui, S. A., Ristow, B., Rahayu, T., Putra, N. S., Widya Yuwono, N., Nisa', K., Mategeko, B., Smetana, S., Saki, M., Nawaz, A., & Nagdalian, A. (2022). Black soldier fly larvae (BSFL) and their affinity for organic waste processing. *Waste Management*, 140, 1-13. <https://doi.org/10.1016/j.wasman.2021.12.044>
- Soetemans, L., Uytendaele, M., & Bastiaens, L. (2020). Characteristics of chitin extracted from black soldier fly in different life stages. *International Journal of Biological Macromolecules*, 165, 3206-3214. <https://doi.org/10.1016/j.ijbiomac.2020.11.041>
- Totakul, P., Matra, M., Sommai, S., & Wanapat, M. (2021). *Cnidioscolus aconitifolius* leaf pellet can manipulate rumen fermentation characteristics and nutrient degradability. *Animal Bioscience*, 34(10), 1607-1615. <https://doi.org/10.5713/ab.20.0833>
- van Huis, A. (2022). Edible insects: Challenges and prospects. *Entomological Research*, 52(4), 161-177. <https://doi.org/10.1111/1748-5967.12582>
- van Soest, P. J., Robertson, J. B., & Lewis, B. A. (1991). Methods for dietary fibre, neutral detergent fibre, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74(10), 3583-3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Vargas-Serna, C. L., Pineda-Osorio, A. N., Gallego-Ocampo, H. L., Plaza-Dorado, J. L., & Ochoa-Martínez, C. I. (2025). Transforming coffee and meat by-products into protein-rich meal via black soldier fly larvae (*Hermetia illucens*). *Sustainability*, 17(2), Article 460. <https://doi.org/10.3390/su17020460>
- Veldkamp, T., Meijer, N., Alleweldt, F., Deruyter, D., van Campenhout, L., Gasco, L., Roos, N., Smetana, S., Fernandes, A., & van der Fels-Klerx, H. J. (2022). Overcoming technical and market barriers to enable sustainable large-scale production and consumption of insect proteins in Europe: A SUSINCHAIN perspective. *Insects*, 13(3), Article 281. <https://doi.org/10.3390/insects13030281>

- Wang, Y., McAllister, T. A., Rode, L. M., Beauchemin, K. A., Morgavi, D. P., Nsereko, V. L., Iwaasa, A. D., & Yang, W. (2001). Effects of an exogenous enzyme preparation on microbial protein synthesis, enzyme activity and attachment to feed in the Rumen Simulation Technique (Rusitec). *British Journal of Nutrition*, 85(3), 325-332. <https://doi.org/10.1079/BJN2000277>
- Wang, Y. S., & Shelomi, M. (2017). Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. *Foods*, 6(10), Article 91. <https://doi.org/10.3390/foods6100091>
- Yang, Y., Shenrui, X., Wenjun, G., Yu, W., Lu, W., & Changhua, L. (2025). Chitinase improves the available energy, amino acids digestibility of black soldier fly and fecal microbiota of growing pigs. *Animal Bioscience*, 38(8), 1733-1745. <https://doi.org/10.5713/ab.24.0920>
- Zou, X., Liu, M., Li, X., Pan, F., Wu, X., Fang, X., Zhou, F., Peng, W., & Tian, W. (2024). Applications of insect nutrition resources in animal production. *Journal of Agriculture and Food Research*, 15 backstage, Article 100966. <https://doi.org/10.1016/j.jafr.2024.100966>

Shotgun Metagenomic and Physicochemical Characterisation of Soil Microbial Communities from the UNISEL Bird Sanctuary, Malaysia

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ABSTRACT

This study explores the microbial diversity within the newly discovered UNISEL Bird Sanctuary using a metagenomics approach. The objective was to establish the first baseline shotgun metagenomic profile of the UNISEL Bird Sanctuary soil microbiome to support future ecological, environmental, and conservation research. Soil samples were subjected to whole-genome shotgun sequencing to characterise microbial community structure and functional potential. Approximately 2.85 million contigs were generated, predicting 3,282,381 genes. Taxonomic profiling using Kraken 2 showed that bacteria represented a major proportion of the community, accounting for approximately 30.01% of clean reads and 34.83% of assembled contigs. A large number of reads remained unclassified, indicating the presence of potentially novel microbial diversity. Functional

annotation successfully classified 44% of coding genes against databases including RefSeq, Swiss-Prot, InterProScan, eggNOG, MEGARes, and KEGG. Comparative metagenomic analysis with publicly available datasets revealed similarities and differences in taxonomic composition, Clusters of Orthologous Groups (COG) classification, and metal resistance genes. The UNISEL Bird Sanctuary soil sample (S3) exhibited higher abundances of copper, iron, and mercury resistance genes, suggesting adaptation

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to specific environmental conditions. This study highlights the importance of documenting microbial diversity for ecosystem health monitoring, ecological understanding, and conservation planning. Further studies involving deeper sequencing and advanced analyses are required to better understand the functions and adaptive mechanisms of uncharacterised microbial communities in this ecosystem.

Keywords: Bioinformatics, metagenomics, microbial diversity, landfills, Whole-Genome Shotgun Sequencing (WGS)

INTRODUCTION

The bird sanctuary has become an increasingly attractive recreational area for tourists and environmentalists to engage in bird-watching activities. This piece of land serves as a home to migratory birds, in which birds are protected and encouraged to breed, as well as promotes the survival and rehabilitation of the birds. In addition, the bird sanctuary area offers natural facilities that serve the conservation of various species and their natural habitat. UNISEL Bird Sanctuary or *Tasik Lindungan Burung* UNISEL is a freshwater-related conservation site found within the Universiti Selangor campus of Selangor, Malaysia. The sanctuary has a mosaic of rehabilitated lakes and other surrounding land habitats created as a result of historical tin mining activities that have evolved through natural ecological succession. These environments support a variety of vegetation and offer significant habitat and stopover for both resident and migratory bird species. The UNISEL Bird Sanctuary is a unique tropical ecosystem to be studied in ecological and environmental studies due to its limited anthropogenic disturbance during recent years. The geochemical legacy of its past mining activity helps to create unique physicochemical characteristics of the soil that affect the dynamics of microorganisms and plant communities. Thus, the sanctuary is an important natural laboratory to investigate soil health, microbial diversity, ecosystem recovery, and preservation of biodiversity in freshwater post-mining landscapes.

Although they are ecologically important, no prior baseline data have been provided on the microbial community structure or functional gene composition of soil at the UNISEL Bird Sanctuary. This information gap restricts the capacity to evaluate the health of the ecosystems, identify the initial signs of environmental stress or pollution, and inform evidence-based conservation and management approaches. Thus, the detailed preliminary study combines physicochemical characterisation and shotgun metagenomic analysis to understand the microbial community structure, functional possibilities, and adaptive characteristics of soil microorganisms in this sanctuary.

Microorganisms play a significant role in mineral recycling and nutrient absorption by flora and fauna in the natural habitat and facilitate many activities, such as the recycling of essential elements and nutrients, biogeochemical cycles, and the development of soil structure (Fulke, Mahajan and Gothawal, 2026). Note that microorganisms are vital in

waste processing, growth-promoting, and the reproduction of plants and animals (Husain, 2022). Hence, exploring microbial communities' profiles and the existing diversity is an interesting scope. For this purpose, metagenomics will be a powerful culture-independent method for studying the microbes and for formulating a microbial diversity profile of uncultured microorganism access as compared to other microorganism profiling methods (Kumar, 2026).

This paper presents our current study of microbial diversity in UNISEL's newly discovered bird sanctuary through a whole-genome sequencing metagenomics approach. Documenting microbial diversity offers numerous benefits, particularly within the context of a bird sanctuary soil metagenomics study. In addition, it provides a baseline for monitoring ecosystem health by identifying the types and abundance of microbes present in the soil, enabling future comparisons to assess the impact of environmental changes or pollution. It also aids in understanding ecosystem functions, as microbial communities play crucial roles in nutrient cycling and decomposition processes. Detailed knowledge of microbial diversity also informs conservation and management strategies (Crawford et al., 2026; Li et al., 2025). As an example, the discovery of unfavourable microbes depletion or an increase in unfavourable microbes enables specific measures to be taken to achieve the restoration of ecological balance. The comparative metagenomics of the study, which compared the soil sample to other datasets, can assist in understanding the differences in the microbial community studied in comparison to other studies, which helps customise the conservation. Also, it can be used in the assessment of the environmental impacts as observed in the research on the copper, iron, and mercury metal resistance gene identification, which suggested potential adaptation to certain environmental factors. Therefore, the abundance and diversity of these genes can be monitored to give information on the amount and effects of the pollutants in the soil.

MATERIALS AND METHODS

Soil Sampling

Sampling was done at three spatially different locations in the UNISEL Bird Sanctuary, Selangor, Malaysia (3°25'52.1" N, 101°26'11.1" E) at a depth of 0 - 20 cm. Sampling sites were identified in undisturbed areas of the sanctuary to capture the indigenous soil microbial communities with minimal anthropogenic impact. Minimal anthropogenic disturbance was the main site selection criteria because the primary goal of this study was to record a baseline soil microbial diversity as a natural heritage and long-term ecological reference. The subsamples obtained were pooled and mixed thoroughly to create one composite soil sample that could represent the soil environment in the sanctuary. The samples of the soils were moved into sterile sampling containers, then moved to the laboratory in insulated ice boxes with ice packs and kept in the freezer at -20 °C until further examination.

Composite sampling has been widely embraced in exploratory soil metagenomics to lessen spatial heterogeneity and create representative baseline microbial profiles, especially in cases where shotgun metagenomic sequencing is constrained by cost. The same methods have been used in previous metagenomics research (Zainun et al., 2018; Xi et al., 2026; Salam & Obayori, 2026; McLaren et al., 2026), which validated its adaptability in measuring baseline biodiversity. Nevertheless, the existence of a single composite samples was used does not allow testing of the fine-scale spatial heterogeneity within the sanctuary; this will be resolved in the future by replicated and spatially stratified sampling designs.

DNA Extraction

A soil sample of UNISEL Bird Sanctuary was used to extract total Deoxyribonucleic Acid (DNA). Sterile tools were used to collect the soil to prevent contamination, and the sample was stored in sterile conditions at 4 °C until it was processed. The DNA extraction was done on the basis of the Qiagen PowerSoil DNA Isolation Kit as per the protocol of the manufacturer (Cat. No. 12888-100). Around 0.25 g of soil was put in a PowerBead Tube, and solution C1 was added to allow cell lysis. Mechanical lysis was performed on the sample by bead-beating to effectively lyse microbial cells. The resultant lysate was centrifuged, and the supernatant was transferred to a clean tube. The solutions C2 and C3 were added sequentially to eliminate inhibitory substances, such as humic acids, and centrifuged. Solution C4 and a silica membrane spin column were used to achieve DNA binding. Solution C5 was used to wash the column to make sure that the DNA is pure, and Solution C6 was used to elute the DNA. The extracted DNA was kept at -20 °C to be used downstream. After extraction, the DNA quantity was measured with Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using the double-stranded DNA (dsDNA) High-Sensitivity (HS) assay. Moreover, quality was checked using agarose gel electrophoresis to confirm the integrity and lack of any major contaminants.

Shotgun Metagenomics Analysis

Shotgun metagenomics analysis is an effective method that is applied to study the taxonomic composition and functional capacity of microbial communities directly using environmental samples. It is also a very detailed study, which involves the sequencing of all the genetic material in a sample. This approach allows detecting a wide spectrum of microorganisms, such as bacteria, archaea, fungi, and viruses, and the characterisation of metabolic pathways and functional genes. Shotgun metagenomics was applied in this study to examine the structure of the microbial community, as well as the functional profiles of the extracted soil DNA.

Data QC and Pre-processing

SolexaQA++ and Bowtie2 were used to perform data quality control and pre-processing. First, the quality of raw sequencing reads was tested with SolexaQA++, which removed low-quality bases to retain high-quality sequences that could be analysed downstream. After that, Bowtie2 was run to align the reads with the PhiX genome, which is a frequent spike-in control in sequencing libraries to eliminate any PhiX contamination. Following the removal of PhiX sequences, paired-end reads were filtered and combined to produce high-quality forward and reverse sequences to be analysed later.

Metagenomics Assembly

Metagenomic assembly was carried out with MetaSPAdes, a dedicated assembler that is designed to assemble microbial genomes with metagenomic data. The resulting high-quality paired-end reads following pre-processing were fed into MetaSPAdes, which takes a multi-step strategy that involves a combination of error correction, graph-based assembly, and iterative refinements to create contiguous sequences (contigs). The assembly that was obtained offered a basis upon which taxonomic and functional analysis could be conducted.

Taxonomic classification and profiling

Kraken 2, a K-mer-based taxonomic classification tool, was used to perform taxonomic classification and profiling. The metagenomic assembly's high-quality reads were matched against the National Centre of Biotechnology Information (NCBI) non-redundant database, which contains extensive genomic reference sequences. Kraken 2 was used to assign taxonomic labels to each read, and this allowed the accurate identification and quantification of microbial taxa in the sample. In line with this, the output was handled to produce taxonomic profiles and relative abundance data, which provided detailed information on the microbial community structure.

Functional Annotation

Analysis of the metagenomic data was conducted through functional annotation of the data using various reference databases and tools to be comprehensive. The annotated sequences were matched to the databases of RefSeq and Swiss-Prot to identify high-confidence protein-coding genes. InterProScan was used to predict protein domains and functional motifs, and eggNOG was used in orthology-based functional assignments and Gene Ontology (GO) classification. Antimicrobial resistance genes were identified using the MEGARes database, which offers information on possible resistance mechanisms. To minimise false-positive assignments, a conservative E-value of 1×10^{-5} was used to cut off searches in BLAST-based searches against RefSeq, Swiss-Prot, and MEGARes.

The reconstruction of metabolic pathways and functional profiling was done through the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, which helped in identifying the microbial metabolic capabilities. The multi-database methodology provided a precise and comprehensive functional characterisation of the microbial community. The use of this multi-database method was selected in particular to avoid the variable coverage rates of environmental metagenomes, which enable the reliability of determining the functional potential core of the soil despite the high number of novel or uncharacterised genes.

Comparative Analysis

Comparative analyses of the metagenomic data produced in this study (S3) with two publicly available datasets, SRR21870203 and SRR21870204, were done to assess the structure of the microbial community and to determine the functional profiles. Kraken 2 was used to taxonomically profile the datasets, and the results were compared to evaluate the diversity and composition of microbial communities. Clusters of Orthologous Groups (COG) analysis was used to provide functional categories that allowed comparison of genes. The frequency of metal resistance genes was compared by aligning annotated sequences of each dataset with the MEGARes database. The Principal Component Analysis (PCA) was conducted to analyse beta diversity to identify the differences between the microbial community structures across the datasets. Also, Canonical Correspondence Analysis (CCA) was applied to measure associations between taxonomic composition and soil physicochemical characteristics, as well as to measure associations between metal resistance genes and soil parameters. These multivariate analyses offered information on environmental and functional drivers of microbial community dynamics among the samples.

RESULTS AND DISCUSSION

Physicochemical Properties of Soil Samples

The main objective of this study was to examine the microbial diversity in the Bird Sanctuary soil of UNISEL (later named S3) using a metagenomics method. The physicochemical composition of the soil was first established to reveal the dynamic interaction between the properties of the soil and the microbial communities that thrived in it. Therefore, the knowledge of this interaction is essential to the evaluation of soil health, ecological role, and the possibility of agricultural or ecological use.

Table 1 provides a summary of the S3 sample physicochemical analysis, supplemented by the heavy metal limits established by the Department of Environment (DOE) Malaysia, which offers a detailed analysis of its chemical, physical, and biological characteristics. The parameters examined are macronutrients, trace metals, toxic elements, Total Organic Carbon (TOC), physical properties, and microbial richness obtained through metagenomic data.

Table 1
UNISEL bird sanctuary soil samples physicochemical results

No.	Parameter	Unit	Concentration	Standard Limit*
1.	Sodium, Na		0.09	
2.	Magnesium, Mg		0.02	675.0
3.	Aluminium, Al	mg/kg	269.972	53,900
4.	Potassium, K		0.12	11.10
5.	Calcium, Ca		0.09	293.0
6.	Chromium, Cr	mg/kg	2.18	14.40
7.	Manganese, Mn	mg/kg	19.36	3.99
8.	Iron, Fe	mg/kg	368.60	44500
9.	Nickel, Ni	mg/kg	1.81	28.90
10.	Copper, Cu	mg/kg	1.06	19.8
11.	Zinc, Zn	mg/kg	3.94	54.3
12.	Arsenic, As	mg/kg	ND<0.OOI	43.0
13.	Selenium, Se	mg/kg	ND<0.02	ND
14.	Silver, Ag	mg/kg	0.32	<0.5
15.	Cadmium, Cd	mg/kg	0.053	0.09
16.	Antimony, Sb	mg/kg	ND<0.OI	4.1E+02
17.	Lead, Pb	mg/kg	8.97	36.0
18.	Phosphorus, P		0.49	
19.	Mercury, Hg	mg/kg	ND<0.OOI	0.42
20.	pH		8.08	
21.	Total Nitrogen as Nitrogen		0.28	
21.	Total Organic Carbon		5.58	
23.	Moisture Content		6.43	

*Source: *Standard heavy metals limit based on the Department of Environment (DOE) Malaysia (2019)

This combined study helps to understand how the soil can be used in relation to environmental and agricultural activities (Eisenhauer et al., 2026). The macronutrient analysis of the soil shows that there are moderate amounts of vital nutrients in the soil, such as total nitrogen (0.28%), phosphorus (0.49%), and potassium (0.12%). Nonetheless, the potassium concentration is much lower than the DOE standard 11.10% indicating that it may require supplementation to facilitate plant growth. Particularly, a deficiency of potassium may impair the growth of plants and decrease their yields, which demonstrates the importance of specialised fertilisation. As Wang et al. (2026) define, potassium is an important macronutrient to plants, and one of the significant factors that inhibits plant growth is the lack of potassium. The lack of potassium in the soil results in a reduction in the size of the leaves, photosynthesis is reduced, and the leaf life is shortened. Severe potassium deficiency may lead to the turning of young leaves to pale yellow, and leaves become smaller and thinner.

The trace metal analysis indicates that the majority of the parameters are within DOE limits. As an example, magnesium (20 mg/kg), aluminium (269.972 mg/kg), and iron (368.60 mg/kg) are much lower than their corresponding limits of 675mg/kg, 53,900mg/kg, and 44,500mg/kg, making the soil safe for ecological use. Other trace metals, including nickel (1.81 mg/kg), copper (1.06 mg/kg), zinc (3.94 mg/kg), and silver (0.32 mg/kg), are well within permissible limits of 28.90 mg/kg, 19.8 mg/kg, 54.3 mg/kg, and 0.5 mg/kg, respectively. At the same time, lead (8.97 mg/kg) and antimony (< 0.01 mg/kg, non-detectable) also comply with DOE limits of 14.40 mg/kg and 410 mg/kg. In addition, toxic elements, including arsenic (< 0.001 mg/kg), selenium (< 0.02 mg/kg), cadmium (0.053 mg/kg), and mercury (< 0.001 mg/kg), are found in insignificant amounts, which cause minimal risks.

Nevertheless, manganese (19.36 mg/kg) is higher than the DOE value of 3.99 mg/kg, and it is concerning that it could cause environmental and agricultural effects. The high concentration of manganese could be explained by the natural sources in the sanctuary ecosystem, such as mineral-rich sediments and organic contributions, such as bird droppings. Furthermore, manganese toxicity could harm plant growth by making it harder for plants to absorb nutrients. Although this is a challenge, metagenomic data show that there are manganese-resistant microbial genes. This indicates that the microbial community of the soil may be essential in reducing the effect of this metal by converting it into less harmful forms. Besides industrial importance, Mn is an important element in the environment. It is one of the most important micronutrients, and almost all microbes require it. Manganese levels are increasing in the ecosystems across the world due to the high levels of industrial activity. Nevertheless, as they possess innate cellular processes that promote homeostasis throughout the ecosystem, bacterial strains play a vital role in the biogeochemical cycling of Mn (Ghosh & Das, 2018).

The TOC content of the soil is 5.58%, which indicates a healthy level of organic matter. Organic carbon plays a vital role in soil fertility, microbial activity and the cycling of nutrients. This high TOC indicates that the sanctuary environment, which is enhanced with organic inputs like bird activity and plant matter, is dynamic and resilient. Moreover, the metagenomic data demonstrated a diversity of microbes such as bacteria, archaea, Eukaryota and viruses as well as genes that are related to the cycling of nutrients, bioremediation and heavy metal resistance. This functional diversity indicates the influence of the physicochemical characteristics of the soil, including nutrient levels and heavy metal content, on the composition and activity of the microbial community (Dincă et al., 2022). The paper correlates physicochemical parameters with microbial functions to reveal the importance of soil chemistry in sustaining microbial diversity and ecological processes. This association is especially important when it comes to the UNISEL Bird Sanctuary, where the ecosystem, through its natural processes, provides inputs to the soil like organic matter through bird activity, which helps in the formation of the soil and its microbial community.

Quality Assessment and Metagenome Assembly Assessment

After filtering, a total of 28,583,695 reads with 62% GC content were obtained from whole-genome shotgun sequencing of the soil sample collected from UNISEL's Bird Sanctuary. The clean reads after the quality assessment were then used for metagenome assembly. It has successfully generated 2,850,843 contiguous sequences (contigs) numbers, with 439,071 for the largest contig, 451 bp for N50, and a total length of 1,245,901,409 bp, as summarised in Table 2.

Table 2
Metagenome assembly statistics

Contig no.	2,850,843
Largest contig	439,071
N50	451
Total length (bp)	1,245,901.409

Taxonomic Profiling

The high-throughput sequencing illustrates the diversity of the microbial community at the phylum level, as presented in the Krona plot in Figure 1. Figure 1(a) demonstrates the taxonomy profiling of microbial diversity in UNISEL's Bird Sanctuary using clean reads, while Figure 1(b) offers the taxonomy profiling based on the assembled data. In this study, clean reads were used directly for taxonomic profiling using Kraken 2 against the nt database. An average of 30.01% bacteria were observed through Kraken classification, with approximately 58.83% unclassified reads. Conversely, taxonomic profiling using assembled contigs showed an average of 34.83% bacteria, of which 51.68% were not classified. The high percentage of unclassified reads suggests the presence of possibly new microbial taxa and genetic functions, which are due to the underrepresentation of tropical soil microbiomes in reference databases and highlight the novelty and exploratory nature of this dataset (Ju & Zhang, 2015).

In the meantime, 8.38% of the reads (preassembly) were determined as Eukaryota, 0.21 as Archaea, 0.05 as viruses, and 2.52 as others. Taxonomic profiling of the post-assembly reads has identified 9.01% of the reads as Eukaryota, 0.25% as Archae, 0.05% as Viruses, and 4.18% as others. The difference between the percentages of bacteria in the clean reads and assembled contigs could be that the process of assembly enhanced the precision of taxonomic profiling. Conversely, assembly is reassembling longer genomic sequences out of smaller random derivatives. This may result in more precise taxonomic profiling, enabling longer and more informative sequences to be identified. It is important to compare the results of taxonomic profiling using clean reads and assembled contigs because they provide different information about the composition of a microbial community (Tran & Phan, 2020).

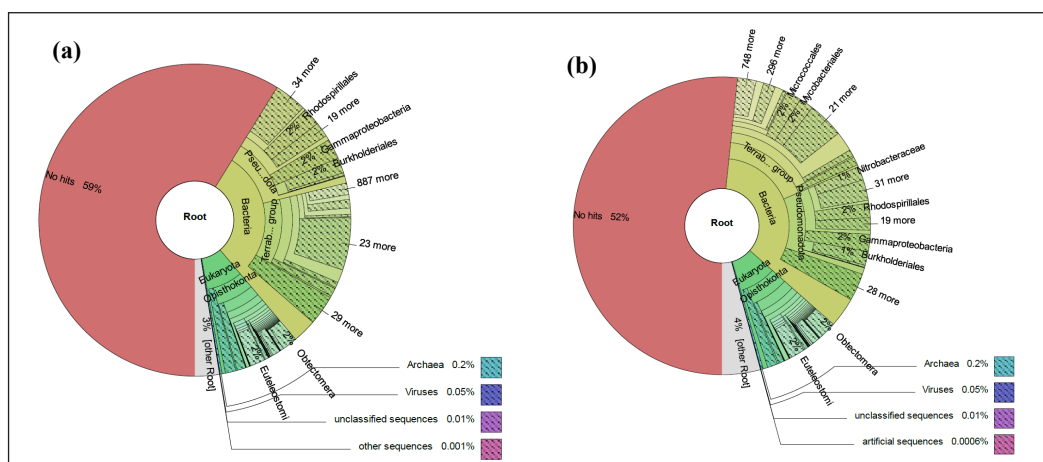


Figure 1. (a) Krona plot of profiled taxonomy of the clean reads (before assembly) against the Kraken 2 database; (b) Krona plot of profiled taxonomy of the assembled contigs against the Kraken 2 database

Kraken 2 is very reliable in identifying known taxa because it has an exact k-mer matching algorithm, which reduces false positives. Nevertheless, the sensitivity of this method is predetermined by the contents of world reference databases. The large percentage of unclassified sequences (51.7% - 58.8%) in this research is a direct result of the large underrepresentation of UNISEL bird sanctuary soil environments in genomic databases at the global and Malaysian levels, in particular. Since tropical freshwater sediments are still significantly underrepresented in metagenomic studies, a significant portion of our data corresponds to new microbial dark matter. This observation emphasises the significance of this preliminary baseline study, and by describing these uncharacterised communities for the first time in a Malaysian sanctuary, we are data mining a baseline that is needed to fill the gap between temperate-centric databases and tropical ecosystem reality.

Gene Prediction and Functional Annotation

The assembled contigs were then used for gene prediction using Prodigal (v2.6.3; -p meta -c -m), successfully identifying 3,282,381 genes with a total base of 348,958,57. The predicted genes were searched against the NCBI Reference Sequence (RefSeq) protein database, Swiss-Prot protein database, InterProScan, EggNOG, MEGARes, and KEGG database. Statistics of the annotations are summarised in Table 3 and Figures 2-5.

Figure 2 visually represents the InterPro annotation (Dimonaco et al., 2022; Hyatt et al., 2013) distribution within the soil metagenome dataset. The figure displays the various functional categories assigned to the predicted genes based on the InterPro databases. The highest contribution of the genes can be observed in the GO term database. This indicates that a significant portion of the predicted genes in the metagenome can be

assigned to broad functional categories related to biological processes, molecular functions, and cellular components. Meanwhile, moderate gene distribution was demonstrated in Superfamily, PANTHER, Gene3D, Pfam, MetaCyc, and Reactome databases, suggesting a substantial representation of genes involved in specific protein families, pathways, and reactions. Low gene distribution was annotated in SMART, CDD, ProSiteProfiles, ProSitePatterns, PRINTS, PIRSF, Coils, and MobiDBLite databases, where the lower representation of genes annotated by these databases could indicate that the functions associated with these specific domains and motifs are less prevalent in the microbial community in the studied area. Other than that, the InterPro annotation distribution provides a glimpse into the functional potential of the microbial community in the soil sample. It determines a diverse range of metabolic capabilities, emphasising functions related to broad GO categories and specific protein families and pathways represented by the moderately distributed databases.

Table 3
Functional annotation statistics against six different databases

Database	No. of Genes	% Genes
RefSeq	2,148,491	65.5
Swiss-Prot	1,176,492	35.8
InterProScan	2,070,549	63.1
eggNOG	2,200,678	67.0
MEGARes	2258	0.07
KEGG	1,024,042	31.2

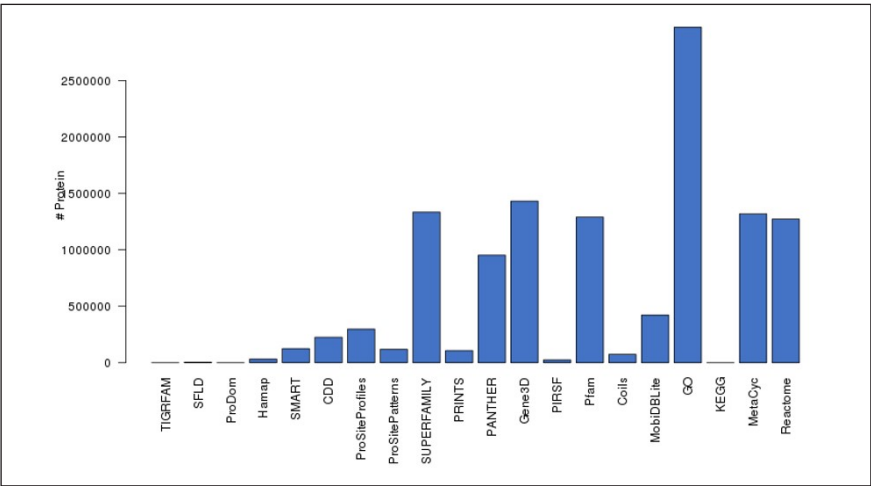


Figure 2. InterPro annotation showing the potential biological functions encoded by the microbial community in the UNISEL's Bird Sanctuary soil

The eggNOG analysis results illustrated in Figure 3 (a) suggest that bacteria are the primary contributors to the genetic diversity and functional potential of the microbial community in this environment. COG analysis, on the other hand, classifies predicted genes into broader functional categories to provide a general overview of the community's capabilities. Figure 3 (b) shows the COG classification analysis, which shows that the greatest percentage of genes is categorised as “Function Unknown.” This indicates that the microbial community in the soil sample contained many genes of unknown function. The large fraction of genes in the COG analysis that are categorised as Function unknown indicates a large gap in knowledge of the functional capabilities of the microbial community. This finding indicates that a significant fraction of the genes in the predicted metagenome of the soil sample does not have a distinct functional attribution according to the existing databases and knowledge.

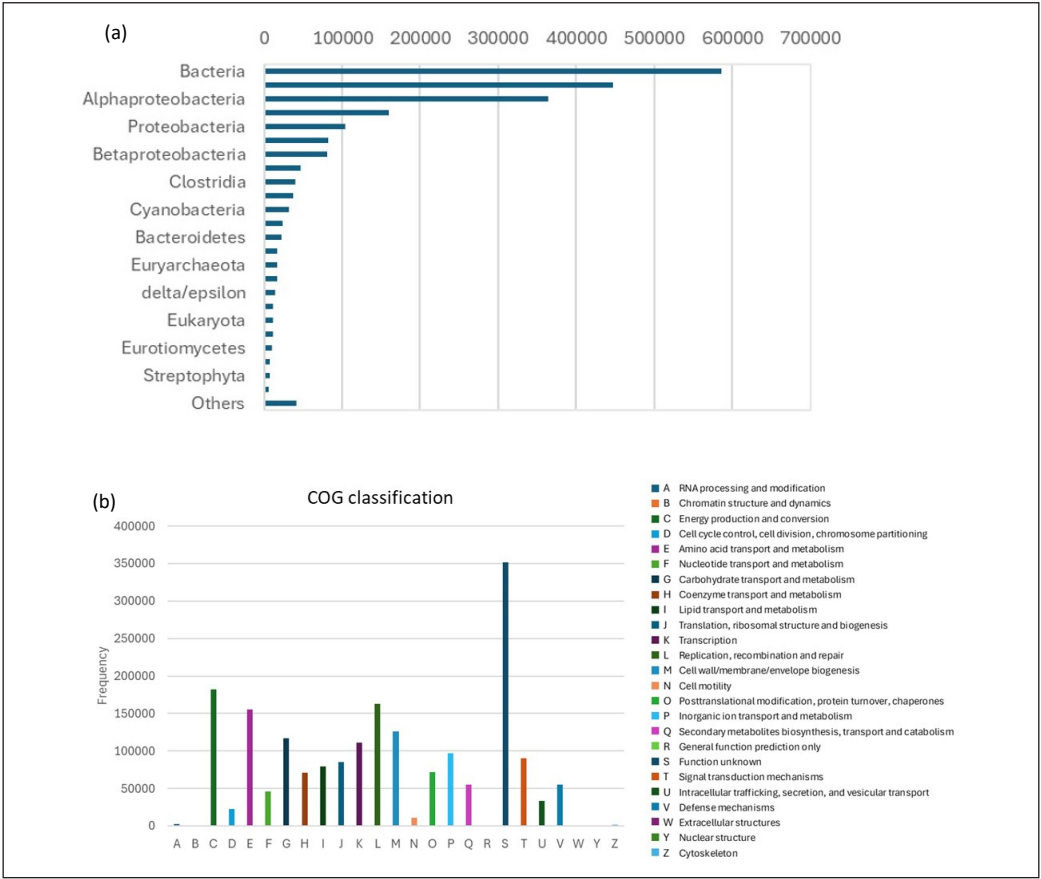


Figure 3. (a) The result from the EggNOG analysis shows the distribution of genes categorised under the bacteria group. (b) COG classification of the predicted genes presents the distribution of proteins based on their orthologous relationships

Likewise, Chen et al. (2022) stated that the role of soil microbial communities in ecosystem processes is essential in predicting how terrestrial ecosystems would act in response to climate change. The high proportion of unclassified sequences in the taxonomic analysis is consistent with the assignment of more than one-third of the predicted genes to 'function unknown' categories, which is in line with the high proportion of unclassified sequences found in the taxonomic analysis. Global functional databases are largely populated with sequences from temperate environments and clinical isolates, leaving tropical freshwater ecosystems like the Malaysian sanctuary soil significantly underrepresented.

The second most abundant gene set in the COG analysis, identified as an “Energy production and conversion” group, indicates that a significant percentage of the microbial community in the lake soil must be engaged in energy-related metabolic activities. This observation emphasises the importance of energy metabolism in the development of the community structure and functioning in this ecosystem (Chen et al., 2022). The third most common gene distribution is categorised as “Replication, recombination and repair”, and the fourth most common as “Amino acid transport and conversion”, which demonstrates some of the basics of the biology and activity of the lake soil microbial community. Essentially, all living organisms need the processes of replication, recombination, and repair to properly duplicate and preserve genetic material (Deng, 2023).

Further analysis of the predicted genes was conducted using MEGARes annotation to classify the presence of genes associated with drug resistance. Figure 4a shows that a significant percentage of genes are in the drug resistance category. This observation implies that the sanctuary soil sample contains a significant pool of genes that might potentially play a role in antimicrobial resistance. Particularly, the presence of these genes may be explained by the use of antibiotics or other antimicrobial agents in the environment. This brings into question the possibility of spreading resistance amongst microbes, which makes it difficult to treat infections caused by the microbes.

According to Table 1, physicochemical analysis indicates that the soil sample contains a high amount of iron. Thus, another annotation on MEGARes was conducted to study the genes around the metal resistance genes, and it showed the predominance of the genes around iron resistance (Figure 4b). The abundance of some metals in the soil sample and the metal resistance genes analysis with MEGARes are good evidence of the adaptation of the microbial community to this particular environment. Furthermore, the number of iron resistance genes is very high, which directly correlates with the high iron levels in the soil. This implies that the microbial community has developed ways of surviving and potentially uses iron, which could otherwise be harmful in high concentrations. According to Doster et al. (2020), MEGARes allow the detection of a wide range of metal resistance genes, which provides a broader understanding of microbial adaptation to the metal population.

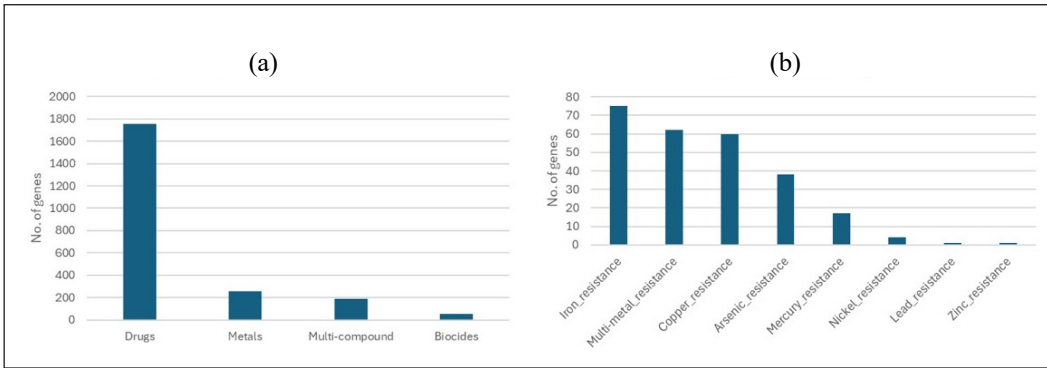


Figure 4. (a) MEGARes annotation of the assembled data of the soil sample, (b) Further MEGARes annotation on the metals-related genes of the soil sample

UNISEL Bird Sanctuary is found on a landscape that is historically related to tin mining operations. This can be seen in the physicochemical outcomes, which show high iron levels in the soil. Functional annotation of the metagenomic data also supported these conclusions by identifying genes linked to metal resistance-related genes, implying adaptation by the microbial adaptation to metal-enriched environments. The analysis of the metagenome data further indicated the most prevalent taxon, *Zygonium ericetorum*, reported to have extreme iron and aluminium stress tolerance (Herburger et al., 2016). The prevalence of these metal-tolerant microorganisms suggests an ecologically relevant role in mediating metal stress, leading to soil stability and allowing vegetation to establish in post-mining environments and could be investigated in future research.

MEGARes annotation also demonstrated that other genes are classified under multi-compound and biocide resistance categories. Multi-compound resistance genes confer resistance to multiple antimicrobial compounds, often with multiple mechanisms of action. These genes might encode efflux pumps that expel various compounds from the cell or enzymes that modify multiple drugs, rendering them ineffective (Maillard, 2018). Notably, biocide resistance genes enable microbes to withstand the effects of biocides, which are chemical agents used to control or kill harmful organisms (Maillard, 2018). These genes can provide resistance mechanisms like reducing biocide uptake, modifying the target site of the biocide, or detoxifying the biocide. According to Maillard (2018), efflux pumps are membrane protein which actively eliminate harmful compounds from cells, such as antibiotics and heavy metals, thus lowering intracellular concentrations and preventing harmful consequences.

Figure 5 shows the distribution of gene annotations according to the KEGG database. The KEGG database gathers manually curated pathway maps that reflect what we know about molecular interactions and reaction networks in biological systems (Kanehisa et al., 2023).

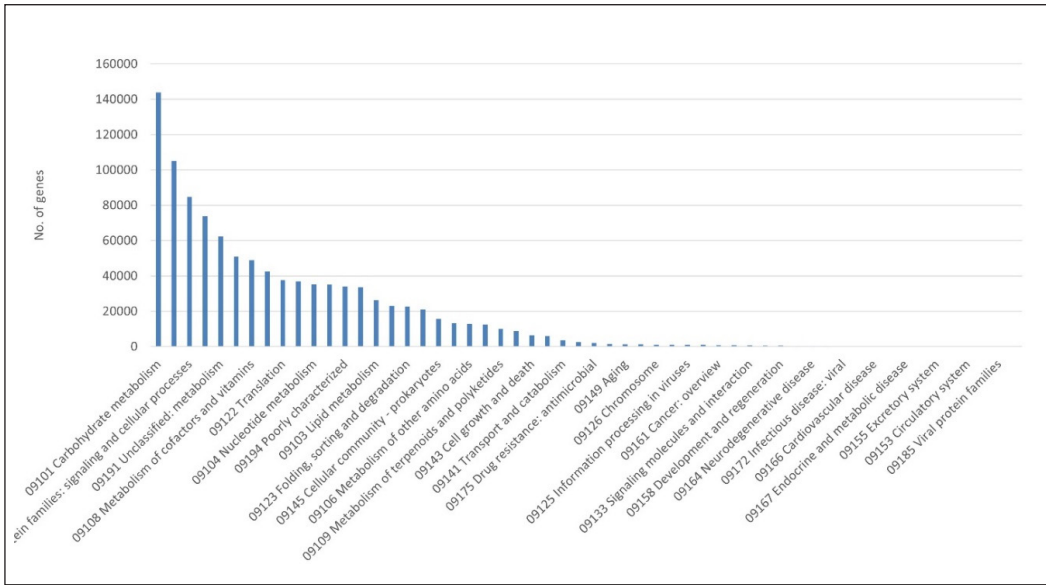


Figure 5. KEGG annotation based on the predicted genes identified in the studied soil sample

The figure is a visual representation of the percentage of genes present in the bird sanctuary soil sample related to different KEGG pathways. A major part of the genes is attributed to "Metabolism," which implies that the microbial community has a substantial role in metabolism. The other major groups are "Genetic Information Processing", "Environmental Information Processing", and "Cellular Processes", indicating the diverse functional ability of the microbial community in the bird sanctuary soil. The outcome of the KEGG analysis, as shown in Figure 5, illustrates the complexity and versatility of the microbial community in the way it performs a variety of biological processes. This involves metabolism, genetic information processing, environmental responses, and cellular functions, and this provides a variety of opportunities to conduct research in future studies.

Comparative Analysis

The UNISEL Bird Sanctuary soil metagenomic dataset was then compared to two publicly accessible NCBI datasets, which was done by a comparative analysis to provide context. These datasets were chosen as they are freshwater-affected soils and offer well-annotated shotgun metagenomic data and can be comparatively analysed taxonomically and functionally. The bird sanctuary soil sample was compared with two public datasets, which were received in BioProject PRJNA889691 and reported by Ren et al. (2017). Henceforth, the soil sample at the Bird Sanctuary of UNISEL will be referred to as S3. Figure 6 shows a phylum-level taxonomy of the three sites (S3, SRR21870203, and SRR21870204),

demonstrating similar and different microbial communities. The common phyla were Actinomycetota, Pseudomonadota, Bacillota, and Bacteroidota, which were all found with varying levels of abundance. The Actinomycetota dominated at S3. Furthermore, the microbial communities at SRR21870203 and SRR21870204 were dominated by Pseudomonadota. This implies that Pseudomonadota proliferation is supported by similar environmental factors or substrates at these locations. Although in varying amounts, Bacillota and Bacteroidota were constantly found at all sites.

Figure 7 shows the COG (Galperin et al., 2025) classification analysis of the three sites (S3, SRR21870203, and SRR21870204), indicating common and distinct functional profiles of microbial communities in the three sites. The three sites are highly represented in the major functional categories, including E (Amino acid transport and metabolism), G (Carbohydrate transport and metabolism), C (Energy production and conversion) and J (Translation, ribosomal structure, and biogenesis), which suggests their core functions in the maintenance of microbial activity. The categories such as R (General function prediction only) and S (Function unknown) are also prevalent, indicating the presence of genes that are either uncharacterised or predictive, which is a common characteristic of metagenomic studies.

S3 has a reduced total abundance of functional genes, indicating a less metabolically diverse community. This is especially noticeable in the categories like N (Cell motility) and T (Signal transduction mechanisms), which show lower representation, meaning fewer cellular interactions and environmental responses. By contrast, SRR21870203 and SRR21870204 show more functional diversity, with increased gene abundances in functional categories like L (Replication, recombination, and repair),

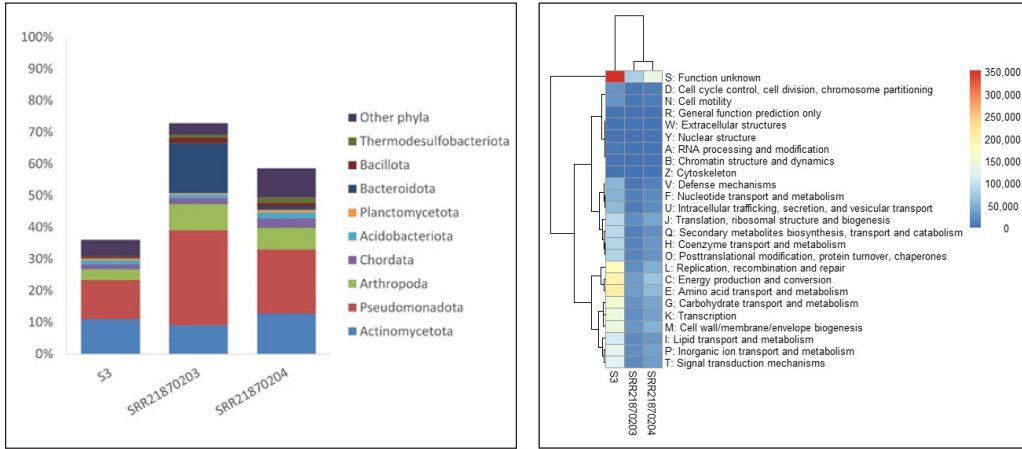


Figure 6. Comparison of the taxonomic profile of phyla in the three samples (S3, SRR21870203 and SRR21870204)

Figure 7. Comparison of COG classification in the three samples (S3, SRR21870203 and SRR21870204)

M (Cell wall/membrane/envelope biogenesis), and V (Defence mechanisms), reflecting more active cellular processes and enhanced adaptability. SRR21870204 also indicates increased representation in D (Cell cycle control, cell division, and chromosome partitioning) and Z (Cytoskeleton), indicating structural and division-related adaptations in this microbial community. Nevertheless, the fact that many uncharacterised genes in S (Function unknown) are highly abundant in S3 contributes to the possibility of new discoveries and the necessity of conducting additional studies to reveal the unknown functions of microbes (Cecil et al., 2018; Price et al., 2018).

Figure 8 shows the outcome of comparing the metal resistance genes in the three samples (S3, SRR21870203, and SRR21870204) using MEGARes annotation. This indicates variations in the prevalence of resistance to different metals due to site-specific environmental factors and possible contamination. Moreover, arsenic, copper, iron, nickel, zinc and lead resistance genes were found in all three samples, suggesting that all three were exposed to these metals, perhaps through contamination or natural geochemical conditions. According to Gillieatt and Coleman (2024), heavy metals are anthropogenic and natural in different sources and highly influential because of their presence in the environment. S3 had the greatest number of multi-metal resistance genes, indicating it was exposed to a combination of metals, with iron resistance genes being the most abundant. Essentially, the difference in the metal resistance genes highlights the importance of the local geochemical environment in shaping microbial populations.

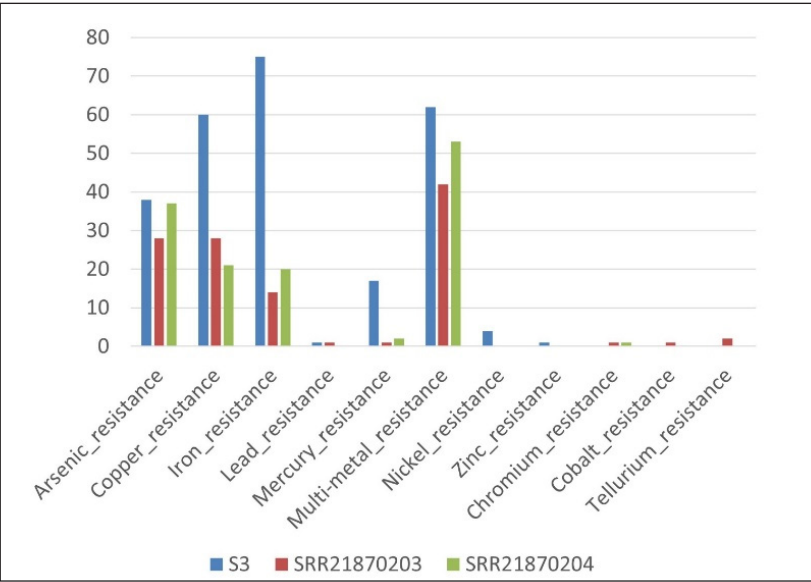


Figure 8. Comparative abundance of metal resistance genes

The PCA plot in Figure 9 represents the beta diversity analysis of microbial community composition across three samples: S3, SRR21870204, and SRR21870203. Beta diversity quantifies the difference in the microbial community composition among samples, and the PCA plot visualises these differences across two principal components (V1 and V2). The sampling distribution pattern shows that there are different microbial compositions (Mikha et al., 2024). Sample S3 is located separately from SRR21870204 and SRR21870203, suggesting a separate microbial community. Also, SRR21870204 is more distant from S3 and SRR21870203, implying that it possesses the most unique microbial composition. Remarkably, S3 is closer to SRR21870203 than to SRR21870204, which means that S3 is more similar to SRR21870203. The differences in microbial diversity observed might be explained by multiple ecological, environmental, or experimental factors. These can be differences in habitat conditions (e.g., soil or water environment), time variability in sampling, or possible technical differences in processing or sequencing procedure (Jiao et al., 2022).

In this research, CCA was used to determine how the microbial taxonomy is related to soil parameters in the three samples (Figure 10) in order to determine the effects of the soil characteristics on different microbial phyla (Bai et al., 2017; Chen et al., 2022; Zhou et al., 2023). The position of the microbial taxa (marked in red) and the three samples with these axes shows the correlation of the microbial communities with particular variables of the soil. SRR21870204 is located far away from SRR21870203 and S3, showing a different microbial composition under unique soil conditions. In contrast, SRR21870203 and S3 are more similar, which implies more similar microbial communities, probably due to similar soil factors. The position of microbial taxa close to a particular sample suggests

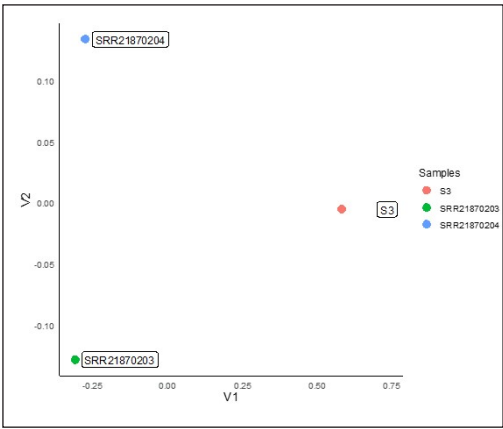


Figure 9. PCA plot (beta-diversity) of taxonomic phyla

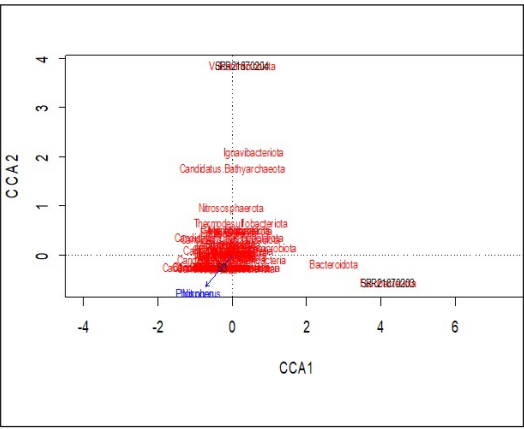


Figure 10. CCA analysis of taxonomy against soil

a close relationship with the microbial community of the sample (He et al., 2023; Sari et al., 2024). In particular, Ignavibacteriota and *Candidatus* Bathyarchaiota are closer to SRR21870204, implying that these phyla are more common in that sample. The blue arrow marked “Phthhokens” is a variable of soil that affects the distribution of microbes. The direction and length of the arrow show how strong this effect is, and the taxa or samples that are closer to the arrow are more affected by this aspect.

The comparative metagenomics indicated similarities and differences between the soil sample and other public datasets, particularly in taxonomic composition, COG classification, and the abundance of metal resistance genes. The increased concentration of copper, iron and mercury resistance genes in the bird sanctuary soil sample indicates potential adaptations to certain environmental conditions. Generally, the metagenomic analysis offers a comprehensive description of the microbial population within the soil, including its diversity and functional attributes.

CONCLUSION

The research gives a detailed baseline characterisation of soil microbial communities in the UNISEL Bird Sanctuary through a combination of shotgun metagenomics and physicochemical methodology. The findings provide a record of the native microbiome of the indigenous soil of a minimally disturbed tropical ecosystem, which will provide useful reference material to future ecological, environmental and conservation research, and also to conservation natural heritage records. The structure of the microbial community and functional profiles were closely correlated with the physicochemical characteristics of the soil, especially mineral composition and indicated the adaptation of microorganisms to geochemical and historical conditions of the site. The abundance of metal-tolerant taxa and related functional genes emphasises the ecological importance of soil microorganisms in ecosystem stability and vegetation establishment in a post-mining landscape.

The metagenomic dataset showed a significant percentage of unclassified reads, which probably explained the underrepresentation of tropical soil microbiomes in the available reference databases, rather than issues with data quality. The observation indicates the novelty of the UNISEL Bird Sanctuary soil microbiome and implies the presence of previously unexplored microbial taxa and genetic functions. These unclassified reads highlight the value of such a dataset as a baseline reference data and demonstrate a large potential in the future of discovery with improved annotation resources and increased sequencing depth. However, additional studies are necessary to investigate the functions and interactions of the uncharacterised microbial components and adaptive mechanisms in this ecosystem, including additional sequencing and other highly advanced analysis methods.

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REFERENCES

- Bai, R., Wang, J., Deng, Y., He, J., Feng, K., & Zhang, L. (2017). Microbial community and functional structure significantly varied among distinct types of paddy soils but responded differently along gradients of soil depth layers. *Frontiers in Microbiology*, 8, Article 945. <https://doi.org/10.3389/fmicb.2017.00945>
- Cecil, J. H., Garcia, D. C., Giannone, R. J., & Michener, J. K. (2018). Rapid, parallel identification of catabolism pathways of lignin-derived aromatic compounds in *Novosphingobium aromaticivorans*. *Applied and Environmental Microbiology*, 84(22), Article e01185-18. <https://doi.org/10.1128/AEM.01185-18>
- Chen, H., Ma, K., Lu, C., Fu, Q., Qiu, Y., Zhao, J., Huang, Y., Yang, Y., Schadt, C. W., & Chen, H. (2022). Functional redundancy in soil microbial community based on metagenomics across the globe. *Frontiers in Microbiology*, 13, Article 878978. <https://doi.org/10.3389/fmicb.2022.878978>
- Crawford, K. M., Dice, C. G., & Clark, G. S. (2026). Why, when, and how microbes can benefit ecological restorations: current approaches and future directions. *New Phytologist*. <https://doi.org/10.1111/nph.70995>
- Deng, S. (2023). The origin of genetic and metabolic systems: Evolutionary structural insights. *Heliyon*, 9(3). <https://doi.org/10.1016/j.heliyon.2023.e14466>
- Department of Environment (DOE) Malaysia (2019). *Contaminated land management and control guidelines no. 1: Malaysian recommended site screening levels for contaminated land*. Ministry of Environment and Water. https://www.doe.gov.my/wp-content/uploads/2021/07/Contaminated-Land-Management-and-Control-Guidelines-No-1_Malaysian-Recommended-Site-Screening-Levels-for-Contaminated-Land.pdf
- Dimonaco N. J., Aubrey W., Kenobi K., Clare A., Creevey C. J. (2022). No one tool to rule them all: Prokaryotic gene prediction tool annotations are highly dependent on the organism of study. *Bioinformatics*, 38(5), 1198-1207. <https://doi.org/10.1093/bioinformatics/btab827>
- Dincă, L. C., Grenni, P., Onet, C., & Onet, A. (2022). Fertilisation and soil microbial community: A review. *Applied Sciences*, 12(3), Article 1198. <https://doi.org/10.3390/app12031198>
- Doster, E., Lakin, S. M., Dean, C. J., Wolfe, C., Young, J. G., Boucher, C., Belk, K. E., Noyes, N. R., & Morley, P. S. (2020). MEGARes 2.0: a database for classification of antimicrobial drug, biocide and metal resistance determinants in metagenomic sequence data. *Nucleic acids research*, 48(D1), D561-D569. <https://doi.org/10.1093/nar/gkz1010>
- Eisenhauer, N., Sünemann, M., Pollierer, M. M., Sun, X., Bardgett, R. D., Bartkowski, B., Delgado-Baquerizo, M., Dirilgen, T., Guerra, C. A., Mathieu, J., Niklaus, P. A., Ristok, C., Seeber, J., Steinwandter, M., Stewart, J. Heijden, M. V. D., Putten, W. V. D. and Potapov, A. (2026). Soil biodiversity effects on ecosystems. *Nature Reviews Biodiversity*, 1-16. <https://www.nature.com/articles/s44358-025-00123-z>
- Fulke, A. B., Mahajan, M. S., & Gothwal, S. K. (2026). Insights on the applicability of metagenomics in marine environments: ecological and biotechnological perspectives. *Marine Biology*, 173(1), 1. <https://doi.org/10.1007/s00227-025-04735-z>

- Galperin, M. Y., Vera Alvarez, R., Karamycheva, S., Makarova, K. S., Wolf, Y. I., Landsman, D., & Koonin, E. V. (2025). COG database update 2024. *Nucleic Acids Research*, 53(D1), D356-D363. <https://doi.org/10.1093/nar/gkae983>
- Ghosh, S., & Das, A. P. (2018). Metagenomic insights into the microbial diversity in manganese-contaminated mine tailings and their role in biogeochemical cycling of manganese. *Scientific Reports*, 8(1), Article 8257. <https://doi.org/10.1038/s41598-018-26311-w>
- Gillicatt, B. F., & Coleman, N. V. (2024). Unravelling the mechanisms of antibiotic and heavy metal resistance co-selection in environmental bacteria. *FEMS Microbiology Reviews*, 48(4), Article fuac017. <https://doi.org/10.1093/femsre/fuac017>
- He, Z., Yuan, C., Chen, P., Rong, Z., Peng, T., Farooq, T. H., Wang, G., Yan, W., & Wang, J. (2023). Soil microbial community composition and diversity analysis under different land use patterns in Taojia River Basin. *Forests*, 14(5), Article 1004. <https://doi.org/10.3390/f14051004>
- Herburger, K., Remias, D., & Holzinger, A. (2016). The green alga *Zygonium ericetorum* (Zygnematophyceae, Charophyta) shows high iron and aluminium tolerance: Protection mechanisms and photosynthetic performance. *FEMS Microbiology Ecology*, 92(8), Article fiw103. <https://doi.org/10.1093/femsec/fiw103>
- Husain, R. Vikram, N., Pandey, S., Yadav, G., Bose, S. K., Ahamad, A., Shami, M., Kumar, K. G., Khan, N.A., Zuhaib, M., & Hussain, T. (2022). Microorganisms: An eco-friendly tools for the waste management and environmental safety. In S. Arora, A. Kumar, S. Ogita, & Y. Y. Yau (Eds.), *Biotechnological innovations for environmental bioremediation* (pp. 949-981). Springer. https://doi.org/10.1007/978-981-16-9001-3_36
- Hyatt, D., Chen, G. L., LoCascio, P. F., Land, M. L., Larimer, F. W., & Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC bioinformatics*, 11(1), 119. <https://doi.org/10.1186/1471-2105-11-119>
- Jiao, S., Chu, H., Zhang, B., Wei, X., Chen, W., & Wei, G. (2022). Linking soil fungi to bacterial community assembly in arid ecosystems. *iMeta*, 1(1), Article e2. <https://doi.org/10.1002/imt2.2>
- Ju, F., & Zhang, T. (2015). Experimental design and bioinformatics analysis for the application of metagenomics in environmental sciences and biotechnology. *Environmental Science & Technology*, 49(21), 12628-12640. <https://doi.org/10.1021/acs.est.5b03719>
- Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M., & Ishiguro-Watanabe, M. (2023). KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Research*, 51(D1), D587-D592. <https://doi.org/10.1093/nar/gkac963>
- Kumar, R. (2026). Isolation and cultivation of novel microorganisms for drug prospecting. In *Bioinformatics, AI, and Machine Learning in Microbial Drug Development* (pp. 3-22). Academic Press. <https://doi.org/10.1016/B978-0-443-33032-2.00012-5>
- Li, L., Ye, J., Yu, M., Jiang, J., Guo, X., Yu, W., & Rong, K. (2025). Dynamic changes in the avian gut microbiome in response to diverse lifestyles. *Ibis*, 167(2), 331-344. <https://doi.org/10.1111/ibi.13388>
- Maillard, J. Y. (2018). Resistance of bacteria to biocides. *Microbiology Spectrum*, 6(2), Article arba-0006-2017. <https://doi.org/10.1128/microbiolspec.ARBA-0006-2017>

- McLaren, M. R., Hershey, O. S., Machtinger, A. N., Rice, D. P., Simas, A. M., Friedman, C. R., Gratalo, D., Philipson, C. W., & Bradshaw, W. J. (2026). Metagenomic sequencing of composite airplane wastewater for surveillance of emerging viruses. *medRxiv*. <https://doi.org/10.64898/2026.01.29.26343714>
- Mikha, M. M., Green, T. R., Untiedt, T. J., & Hergret, G. W. (2024). Land management affects soil structural stability: Multi-index principal component analyses of treatment interactions. *Soil and Tillage Research*, 235, Article 105890. <https://doi.org/10.1016/j.still.2023.105890>
- Price, M., Wetmore, K., Waters, R., Callaghan, M., Ray, J., Liu, H., Kuehl, J., Melnyk, R., Lamson, J., Suh, Y., Carlson, H., Esquivel, Z., Sadeeshkumar, H., Chakraborty, R., Zane, G., Rubin, B., Wall, J., Visel, A., Visel, A., Bristow, J., Blow, M., Arkin, A., Arkin, A., Deutschbauer, A., & Deutschbauer, A. (2018). Mutant phenotypes for thousands of bacterial genes of unknown function. *Nature*, 557(7706), 503-509. <https://doi.org/10.1038/s41586-018-0124-0>
- Ren, Z., Wang, F., Qu, X., Elser, J. J., Liu, Y., & Chu, L. (2017). Taxonomic and functional differences between microbial communities in Qinghai Lake and its input streams. *Frontiers in Microbiology*, 8, Article 2319. <https://doi.org/10.3389/fmicb.2017.02319>
- Salam, L. B., & Obayori, O. S. (2026). Shotgun metagenomics reveals the complete genetic potential for lindane biodegradation in a tropical lentic pond sediment. *International Biodeterioration & Biodegradation*, 206, Article 106206. <https://doi.org/10.1016/j.ibiod.2025.106206>
- Sari, O. F., Bader-El-Den, M., Ince, V., & Arabikhan, F. (2024). *Machine learning approach into bacterial relationship: Exploring 16S rRNA metabarcoding with association rule mining* [Paper presentation]. 2024 IEEE 12th International Conference on Intelligent Systems (IS), Varna, Bulgaria. <https://doi.org/10.1109/IS61756.2024.10705245>
- Tran, Q., & Phan, V. (2020). Assembling reads improves taxonomic classification of species. *Genes*, 11(8), Article 946. <https://doi.org/10.3390/genes11080946>
- Wang, Z., Yao, C., Yang, Y., Segbo, S., Xu, X., Lin, X., Zhou, P., Gao, F., Ni, Z., Shi, T., & Gao, Z. (2026). Effects of foliar potassium fertiliser on photosynthetic capacity and expression of potassium and sugar transporters in peach (*Prunus persica*). *Horticulturae*, 12(3), Article 388. <https://doi.org/10.3390/horticulturae12030388>
- Xi, D., Zhu, F., Zhang, Z., Zhou, S., & Zhang, J. (2026). Forest type shapes soil microbial carbon metabolism: A metagenomic study of subtropical forests on Lushan Mountain. *Microorganisms*, 14(1), Article 220. <https://doi.org/10.3390/microorganisms14010220>
- Zainun, M. Y., & Simarani, K. (2018). Metagenomics profiling for assessing microbial diversity in both active and closed landfills. *Science of the Total Environment*, 616, 269-278. <https://doi.org/10.1016/j.scitotenv.2017.10.266>

Preliminary Study on the Effects of High Dissolved Oxygen on Growth, Survival, and Feeding Rate of *Oreochromis niloticus* (Cichliformes: Oreochromis) using *Egeria densa* as Natural Oxygenator

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ABSTRACT

A major protein source in Malaysia is *Oreochromis niloticus*, and its development is greatly influenced by the level of dissolved oxygen (DO). This research was done to determine the effects of DO on *O. niloticus* development, survival and feeding rate, as well as the potential of *Egeria densa* as a natural oxygenator in place of oxygen pumps. This study hypothesises that *E. densa* serves as a natural oxygenator, enhancing DO levels and promoting growth, survival, and

feeding rate in *O. niloticus*. Three tanks with 50 *O. niloticus* each were assigned as Tank A (control), Tank B (*E. densa*), and Tank C (with an oxygen pump). The fish were fed bran twice daily, with feed amounts adjusted weekly based on mean body weight, over a 12-week experimental period. Survival rate (SR%), weight-gained (WG%), specific growth-rates (SGR%), feed conversion ratio (FCR%) and mortality-rate (MR%) were manually calculated, while the feed utilisation, growth performance, and water parameters were statistically analysed using Minitab 21 and Microsoft Excel. Overall, mean length and weight differed significantly ($p < 0.05$) among tanks ($p = 0.000$).

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In Tank C, length differed significantly between fish with and without oxygen ($p=0.006$), whereas weight did not ($p=0.442$; $p>0.05$). The length-weight regression analysis (LWRs) showed DO has a significant impact on *O. niloticus*' growth rate with negative allometric (Tank A, $b=2.6828$; Tank B, $b=2.5248$), and isometric (Tank C, $b=3.0084$). The highest SR% with the lowest MR% and FCR% was shown in Tank C. *E. densa* respiration competes for DO, making it unsuitable to replace air pumps in aquaculture systems.

Keywords: Dissolved oxygen, *Egeria densa*, growth rate, hyperoxia, *Oreochromis niloticus*

INTRODUCTION

Freshwater aquaculture involves rearing aquatic species in inland waters such as ponds, lakes, rivers, and reservoirs, including brackish water for economic purposes (Li & Liu, 2019). Rising demand for aquaculture protein drives expansion into coastal and offshore areas, enhancing food security and reducing pressure on wild stocks (Gimpel et al., 2018). Fish farming supports economic growth by providing high-quality protein and is often subsidised due to its profitability (Sangirova et al., 2020). According to Kuljanishvili et al. (2021), due to its economic worth and effective production, *Oreochromis niloticus*, or commonly known as Nile tilapia, is the most cultured tilapia species. In Malaysia, this species accounts for 90% production of tilapia as a reasonable protein source (Saba et al., 2020). Its acceptance comes from exponential growth (harvest size in six months), adjustability to changed agricultural practices, high stocking tolerance, and minor protein diet needs, contrasted to marine fish (Mohamad et al., 2021).

According to Nariswari et al. (2020), the growth of *O. niloticus* is affected by stocking density, the rate of feeding, frequency and water quality (DO, temperature, pH and total dissolved solids (TDS)). Lower stocking densities normally improve growth, survival and productivity, making optimal density necessary for maximising production and profits (Abaho et al., 2020). Feed type, ration size and frequency also affect growth and feed conversion ratio of the fish (Mabroke et al., 2021). Knowing feeding patterns advances the fish management, diet formulation and sustainable aquaculture (Tesfahun & Temesgen, 2018). Appropriate feed management ensures economically efficient and eco-friendly production with studies of optimising feeding frequency in clear water systems (Mabroke et al., 2021).

The quality of water is essential for aquaculture, directly influencing fish condition and growth (Mannan et al., 2012). Refer to Ercan et al. (2015) and Sriyasa et al. (2015), the DO, pH, salinity, temperature and TDS are the factors that influence consuming, growth and development, disease tolerance and survival rates of the Nile tilapia. This species thrives at 28 °C-32 °C with a pH of 7-9 and DO above 3 mg L⁻¹ (Nehemia, 2012). Excess phosphorus and nitrogen promote algal blooms, which increase turbidity,

reduce light penetration, limit photosynthesis and gas exchange, and ultimately deplete dissolved oxygen (DO) (Schmidt et al., 2019). Fish survival requires sufficient DO, with 400 ppm as the upper exposure limit for freshwater fish (Munni et al., 2013).

Egeria densa is a freshwater plant native to temperate and subtropical regions of South America. It is unique due to its paired scale leaves, translucent idioblasts, and fiberless leaf margins (Shamso & Hosni, 2020). Currently, it is found worldwide except in Antarctica (Drexler et al., 2021), which can grow up to 5 meters long in water up to 4 meters deep. It has trailing stems that produce roots regularly with oval to oblong leaves, 1.5-4 cm long, that grow in clusters of 4-5 along the stems. Lower leaves appear in pairs or groups of three, while upper leaves form clusters of 4-8, creating dense tips that reach the water's surface (Yarrow et al., 2009). It thrives in lakes, ponds, and slow-moving waters, usually at depths of 0.1-1.5 meters (Białowiec et al., 2019). *E. densa* was introduced as an oxygenator before becoming an invasive species (Shamso & Hosni, 2020). In order to survive in low CO₂ environments, it uses Phosphoenolpyruvate carboxylase (PEPC) to efficiently take up bicarbonate (HCO₃) (Lara et al., 2002).

To increase oxygen levels and improve water quality, aeration of freshwater is vital (Febiyanto, 2020). Circulating water and dissolved oxygen levels must be maintained during live fish transport, whereas in standing water, aeration aids in oxygen balance (Rifat et al., 2019; Yao et al., 2022). In fish farming, the aeration systems include paddle wheels and spiral aerators (Tanveer et al., 2018). Expanding the water surface enhances oxygen intake, while water mixing ensures even distribution and prevents over-oxygenation. Mixing during the day helps conserve oxygen for nighttime use (Rifat et al., 2019).

This study aims to demonstrate the importance of oxygen levels in fish growth, survival, and feeding rates, specifically in *Oreochromis niloticus*. The findings may indirectly impact farming and fishing income. Hence, the objectives of this study are to assess the effect of high oxygen levels on the growth, survival, and feeding rate of *Oreochromis niloticus* and to evaluate the effectiveness of *Egeria densa* as a natural oxygenator in replacing oxygen pumps in aquaculture.

MATERIALS AND METHODS

Experimental Designs and Sample Identification

The experiment was conducted at Kolej Seri Menanti 6, Kuala Pilah campus, UiTM Negeri Sembilan from November 11, 2021, to January 19, 2022. Three 500-gallon circular tanks (60-inch top diameter, 52-inch bottom diameter, 51-inch height) were filled with 2000 litres of tap water using 120 ml of anti-chlorine. The tanks labelled A, B, and C were placed outdoors under sunlight and covered with a black net for fish protection.

A total of 150 *Oreochromis niloticus* fry were purchased from a supplier in Kg Beting, Ulu Muar district. Their identification followed Tilapia and Trout (Alam et al., 2017) and

Zhu et al. (2016), using caudal fin banding and body colour patterns. Nile tilapia have distinct vertical bands on the caudal fin and a pink or grey throat (Alam et al., 2017). Each tank received 50 fry for observation. Their initial size ranged from 3.02-3.67 cm in standard length (SL), 1.4-1.6 cm in body depth (BD), and 1.4-2.4 g in weight (W). Table 1 provides characteristics of each experimental tank as refer Figure 1, Figure 2 and Figure 3 in this study. In Tank B, 150 bunches (200 grams) of *E. densa* were added, and the same amount was used throughout the experiment to ensure consistency.

Ten *O. niloticus* were randomly selected from each tank and measured twice weekly for SL, BD, and weight using a ruler and a digital scale for 12 weeks. A pre-weighed plastic beaker containing 500 mL of water was used to weigh fish, thereby minimising handling stress. The morphometric measurements were illustrated in Figure 4. The snout tip to the caudal fin base measurement was taken for SL measurement, while the maximum vertical distance between the dorsal and ventral margins was measured for BD measurement (Jayraj et al., 2019).

In order to record mortality rates and fish survival, the tanks were monitored daily. To ensure consistent water quality across the treatments, the DO Meter (JPB 70 A) and pH/TDS meter were used to record temperature, DO, TDS, and pH. Readings were taken thrice weekly in the morning, afternoon, and evening (weeks 1 to 12). From weeks 10-12, Tank C's oxygen supply was cut off, and observations continued.

Table 1
Characteristics of each experimental tank in the study (11 November 2021 - 19 January 2022)

Tank		Components
Tank A	Treated water + <i>O. niloticus</i>	
Tank B	Treated water + <i>Egeria densa</i> (150 bunches) + <i>O. niloticus</i>	
Tank C	Treated water + oxygen pump and filter + <i>O. niloticus</i>	



Figure 1. Tank A (control) containing treated water and *O. niloticus*



Figure 2. Tank B containing treated water, *E. densa* (150 bunches) and *O. niloticus*



Figure 3. Tank C equipped with an oxygen pump and filter, containing treated water and *O. niloticus*

Feeding Management

O. niloticus was fed bran twice daily at 0900 and 1700. Fry received crumble feed, while fingerlings were given floating pellets. Different feed sizes were used to suit the mouth size and feeding habits of fry and fingerlings, and floating bran is easy to collect for weighing, allowing fair comparison of feed conversion. The feed amount was calculated using the feed ration formula and adjusted weekly based on average fish weight. The feeding rate (%) followed the FAO (2021) schedule (Table 2). Leftover feed was removed after an hour, dried, and used to calculate the feed conversion ratio (FCR) using Equation 1 for each tank.

$$\text{Feed ration} = \text{average fish size (weight)} \times \text{feed rate (\%)} \times \text{total number of fish} \quad [1]$$

Table 2
Feeding rate (%) of Oreochromis niloticus during fry, fingerling, juvenile and grower stages

Life Stage	Fish Size (g)	Feeding Rate (% body weight)
Fry	1 - 5	10 - 6
Fingerling	5 - 20	6 - 4
Juvenile	20 - 100	4 - 3
Grower	100 - 250	3 - 2
	> 250	2 - 1.5

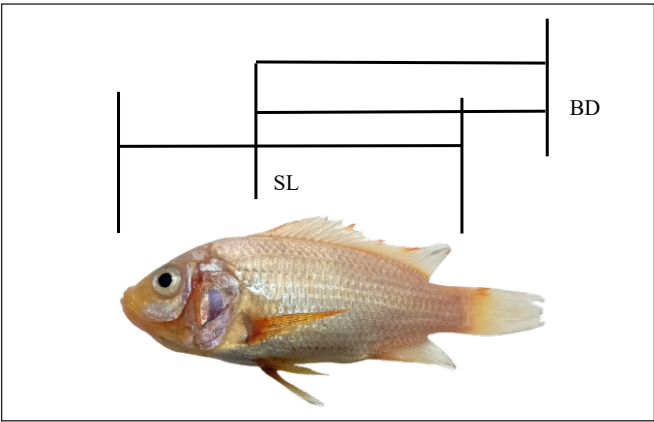


Figure 4. Measurement of standard SL, BD, and W of *O. niloticus*

Data Analysis

The survival rate (SR%), weight gained (WG%), specific growth rates (SGR%), feed conversion ratio (FCR%), and mortality rate (MR%) can be expressed by the Equations 2, 3, 4, 5, and 6. These were used to assess growth performance and feed utilisation.

$$SR\% = \left(\frac{\text{Number of fish at the end of the experiment}}{\text{Number of fish at the beginning of the experiment}} \right) \times 100$$
 [2]

$$WG\% = \left[\frac{\text{Final weight, g} - \text{Initial weight, g}}{\text{Initial weight, g}} \right] \times 100$$
 [3]

$$SGR\% = \left[\frac{\text{Final weight, g} - \text{Initial weight, g}}{\text{No. of day}} \right] \times 100$$
 [4]

$$FCR\% = \left[\frac{\text{Feed fed (g)}}{\text{Weight gained (g)}} \right] \times 100$$
 [5]

$$\text{MR}\% = \left[\frac{\text{Number of dead fish}}{\text{Total number of fish at the beginning of the experiment}} \right] \times 100 \quad [6]$$

Fish length and weight recorded after 11 weeks were tabulated. The length-weight relationship (L, cm) and weight (W, g) were calculated using the power regression equation $W = a L^b$, where a is the intercept and b is the regression coefficient. The relationship coefficient (r^2) determined the association between length and weight. Growth was assessed based on b : isometric growth ($b=3$), positive allometric growth ($b>3$), and negative allometric growth ($b<3$) (Morey et al., 2003).

2-sample-t-test and One-way ANOVA were used to analyse the effect of DO on fish growth and feed efficiency in *O. niloticus*. This analysis was performed using the Minitab 21 software and was considered at a significance level of 5% ($P<0.05$). Microsoft Excel was used to save the data and create graphs.

RESULTS

Dissolved Oxygen Measurements

Tank C had the highest DO level (12.32 ± 3.83^c mg/L), followed by Tank B (10.84 ± 3.06^b mg/L) and Tank A (8.82 ± 2.23^a mg/L), with a 3.52 mg/L difference between the highest and lowest levels. The analysis shows significant variation in DO levels across tanks, but the data's accuracy is limited as it was collected only during the daytime.

Mean Length and Weight of Treated and Untreated *Oreochromis niloticus*

Figures 5 and 6 compare the mean length (cm) and weight (g) of *O. niloticus* in Tank A (control), Tank B, and Tank C. The mean length of fish in Tank A (6.157 cm) and Tank B (5.7463 cm) showed no significant difference ($p=0.055$; $p>0.05$). However, Tank C (7.089 cm) had a significantly higher mean length ($p=0.00$; $p<0.05$) compared to both Tank A and Tank B. Similarly, fish weight in Tank A (9.095 g) and Tank B (7.08 g) showed no significant difference ($p=0.05$; $p>0.05$), but Tank C (15.701 g) had a significantly higher weight ($p=0.00$; $p<0.05$). Overall, there were significant differences in the mean length ($p=0.000$, $p<0.05$) and mean weight ($p=0.000$, $p<0.05$) of the fish.

To highlight the importance of DO, the oxygen supply in Tank C was cut off for three weeks. The mean length and weight of *O. niloticus* were then compared to the same samples when oxygen was supplied in the previous three weeks. Figures 7 and 8 show the mean length and weight ranges in Tank C with oxygen (TCO) and without oxygen (TCWO). The mean length and weight range in TCO (0.71 cm; 4.65 g) were higher than in TCWO (0.33 cm; 3.02 g).

There was a significant difference in mean length ($p=0.006$, $p<0.05$), but no significant difference in mean weight ($p=0.442$, $p>0.05$). Despite the lack of a significant difference, a decrease in weight was observed, indicating a possible effect of dissolved oxygen.

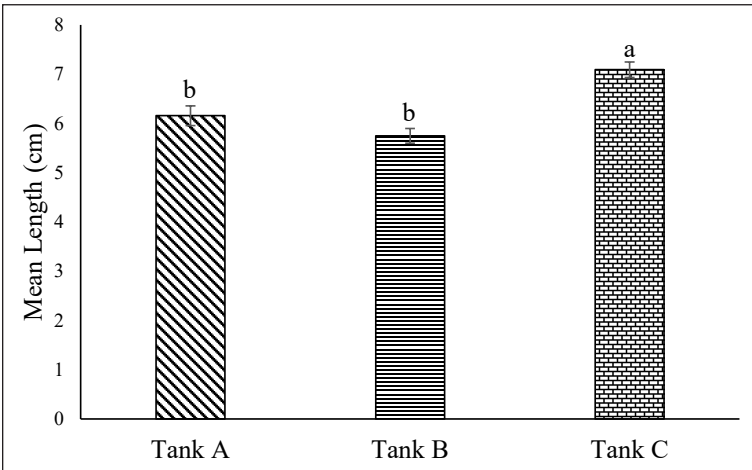


Figure 5. Comparisons between Tank A, Tank B and Tank C for the mean length (cm) of *Oreochromis niloticus* Note. Tank A = Control; Tank B = Treatment 1 (*Egeria densa*); Tank C = Treatment 2 (supplied with oxygen); Means ($\pm$ SE) marked with different letters “a” and “b” are significantly different at $p=0.05$ (Tukey’s test), confidence level=95%

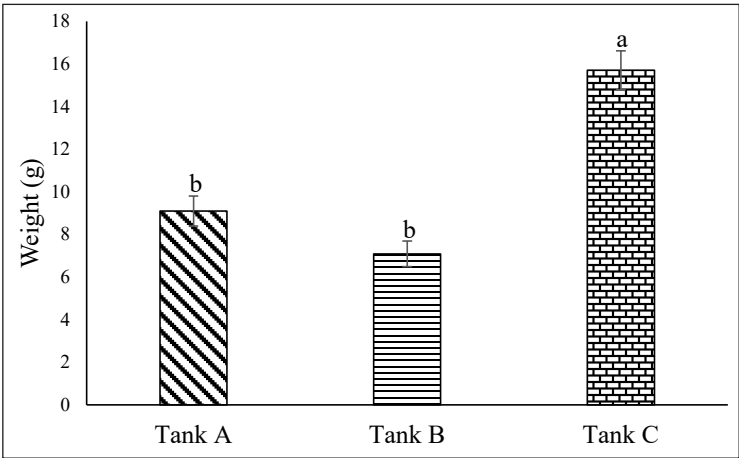


Figure 6. Comparisons between Tank A, Tank B and Tank C for the weight (g) of *Oreochromis niloticus* Note. Tank A = Control; Tank B = Treatment 1 (*Egeria densa*); Tank C = Treatment 2 (supplied with oxygen); Means ($\pm$ SE) marked with different letters “a” and “b” are significantly different at $p=0.05$ (Tukey’s test), confidence level = 95%

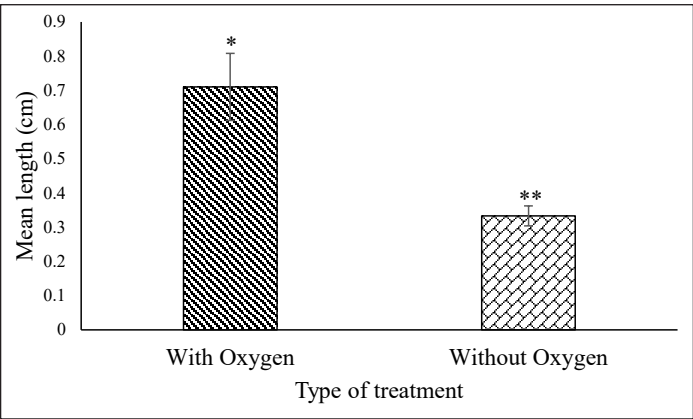


Figure 7. Mean length of *Oreochromis niloticus* in Tank C with and without oxygen
Note. Bars represent mean $\pm$ SE. Asterisk (*) indicates a statistically significant difference between groups at $p<0.05$ (Tukey’s test). cm = in centimetres

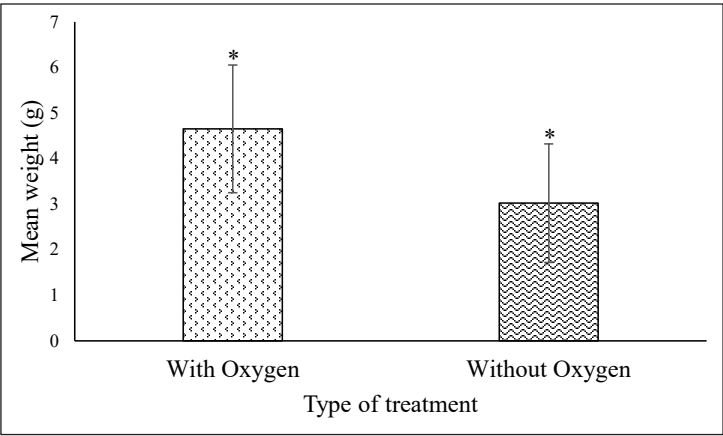


Figure 8. Mean weight of *Oreochromis niloticus* in Tank C with and without oxygen
Note. Bars represent mean $\pm$ SE. Asterisk (*) indicates a statistically significant difference between groups at $p<0.05$ (Tukey’s test). g = in grams

Length-weight Regression Analysis of *Oreochromis niloticus*

Figures 9, 10, and 11 show the length-weight (LW) regression analysis of *O. niloticus* in Tanks A, B, and C. The fish in Tanks A and B exhibited negative allometric growth, with b values 2.6828 and 2.5248 ($b<3$), respectively. Oppositely, Tank C showed isometric growth, with $b=3.0084$ ($b=3$). The LW relationship equations were $W=0.061 \times L^{2.6828}$ ($R^2=0.9175$) for Tank A, $W=0.0779 \times L^{2.5248}$ ($R^2=0.9177$) for Tank B, and $W=0.0348 \times L^{3.0084}$ ($R^2=0.962$) for Tank C. These equations help determine the relationship between fish length and weight.

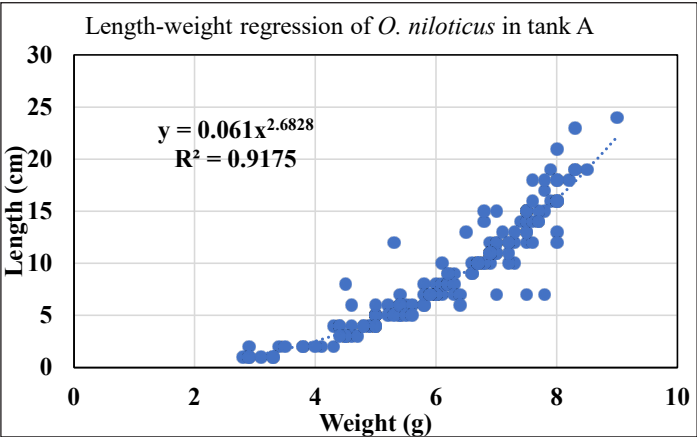


Figure 9. Length-weight regression of *Oreochromis niloticus* in Tank A (control) shows negative allometric growth ($b < 3$)

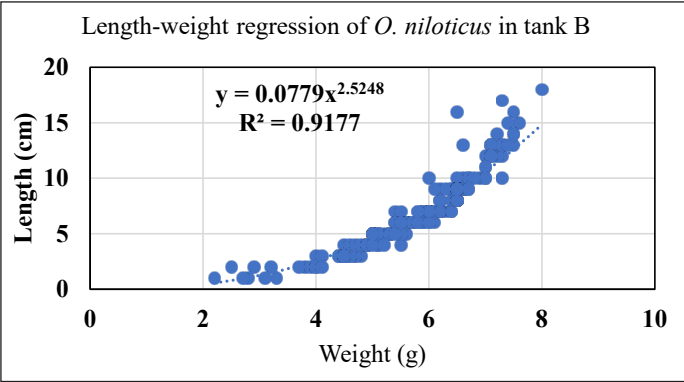


Figure 10. Length-weight regression of *Oreochromis niloticus* in Tank B (*Egeria densa*) shows negative allometric growth ($b < 3$)

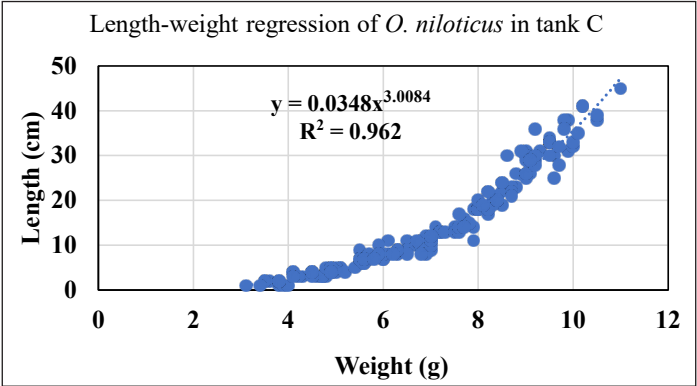


Figure 11. Length-weight regression of *Oreochromis niloticus* in Tank C (aerator) shows isometric growth ($b = 3$)

Survival and Death Rate, Weight Gained, Specific Growth Rate and Feed Conversion Ratio of *Oreochromis niloticus*

Table 3 shows the survival rate (SR%), weight gain (WG%), specific growth rate (SGR%), feed conversion ratio (FCR%) and death rate (MR%) of *O. niloticus* in Tank A, Tank B, and Tank C. *Oreochromis niloticus* in both Tank A and Tank C show the highest SR% (100.00 ± 0.00) and 0.00% for MR%, respectively. Differently in Tank B, the lowest SR% (98.00 ± 1.41) and highest MR% (2.00%) were recorded as one of the fish unable to survive during the experiment. In the present study, the best FCR% was in Tank C with 6.50 ± 5.88 , and the lowest was in Tank B (11.20 ± 6.92). WG% of *O. niloticus* in Tank A (120.54 ± 0.61), tank B (71.67 ± 1.07) and Tank C (140.63 ± 5.23) showed significant differences ($p>0.05$) and were separated into different groups. WG% was highest in Tank C in the present study, with the lowest FCR%. The specific growth rate (SGR%) measures the daily percentage increase in fish weight (Lugert et al., 2016). The SGR% recorded was 1.54 ± 0.27 in Tank A, 2.41 ± 0.04 in Tank B, and the highest at 4.82 ± 0.37 in Tank C. A significant difference was observed between the treatments and the control.

The values, $n = 65$, are given as mean + standard deviation. In a column, different superscript letters ^{a,b,c} denote significant differences across treatments ($p<0.05$).

Table 3
Oreochromis niloticus survival rate, mortality rate, weight gained, specific growth rates and feed conversion ratio in Tank A (control), Tank B (*E. densa*) and Tank C (O_2 pump)

Parameter	Treatments		
	Tank A (control)	Tank B (<i>E. densa</i>)	Tank C (O_2 pump)
SR%	100.00 ± 0.00^a	98.00 ± 1.41^a	100 ± 0.00^a
WG%	120.54 ± 0.6^b	71.67 ± 1.07^c	140.63 ± 5.23^a
SGR%	2.41 ± 0.04^b	1.54 ± 0.27^c	4.82 ± 0.37^a
FCR%	8.00 ± 2.42^b	11.20 ± 6.92^c	6.50 ± 5.88^a
MR%	$0.00\% ^a$	$2.00\% ^a$	$0.00\% ^a$

Note. SR% = survival rate; WG%= weight gained; SGR% = specific growth rates; FCR% = feed conversion ratio; MR% = mortality rate; O_2 = oxygen; *E. densa* = *Egeria densa*

DISCUSSION

Effects of Dissolved Oxygen towards the Growth of *Oreochromis niloticus*

DO is crucial in aquaculture as fish absorb oxygen passively through their gills (Valverde et al., 2006). It comes from air diffusion and photosynthesis by chlorophyll-containing organisms (Abdel-Tawwab et al., 2015). Oxygen transfer depends on a concentration gradient, requiring adequate DO levels (Yildiz et al., 2017).

Limitations of this study include the potential for *E. densa* to introduce confounding factors beyond oxygenation, as the plant may compete with fish for available nutrients, potentially affecting growth and feeding performance. Nighttime measurement of DO levels also could not be carried out due to safety concerns at the study site.

Dissolved Oxygen was monitored three times per week at different times, which are during morning, midday, and afternoon. The recorded values were averaged before being analysed. DO concentrations ranged from 8.82 to 12.32 mg/L, which appears to fall within the typical range. Healthy water typically has DO above 5.0 mg/L (Rouf et al., 2022), while levels below 1-2 mg/L can be fatal (Rouf et al., 2022). Fish growth declines when DO is between 3-5 mg/L (Stickney, 2017). If DO drops below 3 mg/L, feeding should stop, and corrective actions should be taken. Tilapia require at least 2.0 mg/L for smaller fish (60-100g) and 5.0 mg/L for larger fish (200-270g) (Saravanan et al., 2012). Atlantic salmon, however, cannot survive in less than 6 mg/L, as this is considered hypoxic (Alver et al., 2022).

This study confirms that oxygen is a crucial abiotic factor affecting fish development, including *O. niloticus*, where low DO levels negatively impact length and weight. Higher DO levels in Tank C likely contributed to better fish growth, supporting findings from Abdel-Tawwab et al. (2015). This is further validated by the length-weight regression analysis as the exponent b value indicates growth patterns with $b < 3$ negative allometric growth (fish grow longer but remain lean), $b = 3$ isometric growth (length and weight increase proportionally), and $b > 3$ signifies positive allometric growth (fish become stouter as they grow) (García-Fernández et al., 2020).

Lentic waters with low DO impede fish growth. *O. niloticus* in Tank C showed isometric growth ($b = 3.0084$, $b > 3$), proving that increased DO from aeration (3.2 mg/L) boosted growth compared to tank A. Lakani et al. (2013) also observed lower body weight in fish reared in normal and low oxygen conditions compared to those in high oxygen environments. Comparably, halibut (*Hippoglossus hippoglossus* L.) growth gradually increased as oxygen saturation rose from 57% to 100% (Thorarensen et al., 2010). Following the cessation of oxygen supply to Tank C's water pump, growth rate declined, aligning with Franklin (2014), who asserts that DO is influenced by the re-aeration, which is a key factor in fish habitat quality and stream health. Other studies show comparable findings, such as channel catfish, *Ictalurus punctatus* (Buentello et al., 2000), spotted wolfish, *Anarhichas minor* (Foss et al., 2002), striped bass, *Morone saxatilis* (Brandt et al., 2009), Atlantic halibut, *Hippoglossus hippoglossus* (Thorarensen et al., 2010), Japanese flounder, *Paralichthys olivaceus* (Duan et al., 2011), grass carp, *Ctenopharyngodon idella* (Gan et al., 2013) and Nile tilapia, *O. niloticus* (Abdel-Tawwab et al., 2014).

DO levels are regulated by production (photosynthesis) and consumption (respiration, organic matter breakdown, and oxygen demand) (Banerjee et al., 2019).

Goodwin et al. (2008) stated DO is produced during the day through photosynthesis but continuously consumed by respiration, causing fluctuations. Despite *E. densa* supplying oxygen in Tank B, fish growth remained negative allometric. This is due to competition for DO between *O. niloticus* and *E. densa*, especially during nighttime when oxygen levels decline under dense macrophyte cover, resulting in hypoxic conditions (Ribaud et al., 2014). According to Białowiec et al. (2019), *E. densa* increased oxygen levels during daylight and then shifted to oxygen consumption during the night due to respiration. The respiratory activity of *O. niloticus* and *E. densa* at night likely contributed to a significant reduction in DO levels in Tank B, which may have negatively affected the growth performance.

Fish in Tank A (control) had significantly lower growth than those in Tank C. Lotic waters (streams, rivers) dissolve more oxygen than lentic waters (ponds, lakes) (McDonald, 2012). The lentic condition in Tank A reduced DO levels, limiting *O. niloticus* growth. As a result, fish in this tank exhibited negative allometric growth due to insufficient oxygen for movement, feeding, and metabolism. As fish grow, their oxygen demand increases faster than their ability to absorb oxygen, leading to abnormal growth due to the disproportionate expansion of body volume over surface area (Machado, 2019). This study supports findings by Mallya (2007), which reported that low DO levels reduce food intake and decelerate fish growth. When DO declines, fish exhibit aquatic surface respiration (ASR) where they swim to the surface for oxygen-rich water (Franklin, 2014). This behaviour was often observed in Tanks A and B.

Comparing the effects of additional DO and natural DO shows that both significantly impact fish growth. These findings confirm previous studies suggesting that optimal growth occurs at oxygen saturation levels of around 100% or greater (Domenici et al., 2017). In this study, fish in the control tank without an oxygen supply were significantly smaller in size than those in the hyperoxia. Poor growth under low DO conditions is likely due to inadequate oxygen availability for crucial metabolic processes.

Effects of Dissolved Oxygen on the Survivability of *Oreochromis niloticus*

DO is crucial for fish growth, and consistently low levels can reduce feed intake, growth, and feed efficiency (Li et al., 2020). Most post-larval fish absorb oxygen through their gills (Cox, 2003). When DO drops below the required threshold, fish strive to convert energy efficiently, leading to slower growth, feeble swimming, and lower feed efficiency (Roslan et al., 2021). In response, fish intensify opercular ventilation and gasp for air. Abdel-Tawwab et al. (2014) found that low DO significantly slowed Nile tilapia growth by reducing feed intake due to poor appetite and digestion.

This experiment examined DO's impact on *Oreochromis niloticus* survival rate (SR%) and feed conversion efficiency (FCR%). Tanks A and C had the highest SR% with no recorded deaths,

while Tank B had the lowest SR% and highest mortality rate (MR%). Deaths in Tank B occurred due to fish caught up in *E. densa*, preventing feeding and depleting energy. The struggle to escape increased metabolism and exhaustion of the fish. Maharani et al. (2022) noted that survival rates are influenced by feeding frequency, feed-to-weight ratio, and feed quality, which indirectly affected the SR% in this study. Therefore, DO may not be the key factor impacting *O. niloticus* survival in this experiment.

Survival rate measures the percentage of organisms remaining alive over time, affected by water quality, feed and proper maintenance. Ahmed et al. (2013) recorded Tilapia survival rates of 82-90%, aligning with this study. Mortality rate, which includes natural and fishing mortality, reflects fish population loss (Abu El-Nasr, 2021). Natural mortality results from predation, disease, pollution, or environmental factors (Allen & Hightower, 2010), while fishing mortality happens due to being caught by various gear (Sparre et al., 1989).

According to the United States Agency for International Development (2011), the feed conversion ratio (FCR) measures the amount of feed required for fish growth. A lower FCR% indicates better feed efficiency and a diet quality (Iskandar & Elrifadah, 2015; USAID, 2011). Adequate DO spikes fish activity and digestion, resulting in higher feed intake and more efficient growth. Mengistu et al. (2019) reported a positive correlation between feeding rate and DO, indicating that increasing DO from the lowest to the highest levels improved FCR by 50%. Tank C had the lowest FCR%, suggesting optimal water quality for feeding, while Tank B showed the highest FCR%. However, the large standard deviation probably indicates variations in growth among individuals and feed intake. The mean FCR still represents the treatment's feed conversion efficiency. Elvy et al. (2022) noted that a lower FCR% is linked to reduced energy needs for digestion. Research by Abdel-Tawwab et al. (2015), Bergheim et al. (2006), and Duan et al. (2011) confirmed that oxygen availability and diet affect fish growth rate and feed efficiency.

FCR%, WG%, and SGR% are closely related, as noted by Brandt et al. (2009) and Duan et al. (2011). Specific growth rate (SGR%) measures the daily weight gain percentage of fish (Lugert et al., 2016). Higher WG% and SGR% in a hyperoxic environment suggest increased feed intake due to better digestion and nutrient absorption (Gan et al., 2013; Tran-Duy et al., 2008). In contrast, fish in low DO conditions consumed less feed, had the highest FCR, and showed poor growth. Adequate DO assists fish in reducing energy expenditure, permitting more energy for growth and fat storage (Duan et al., 2011). Growth rate is also influenced by supplemental feeding, sex, stocking density, and water temperature (Mizanur et al., 2014). This study also demonstrated a correlation between SGR% and WG%, with Tank C displaying the highest and Tank B the lowest values for both parameters.

In our opinion, SGR% was lowest in Tank B despite the presence of *E. densa*, which provides oxygen through photosynthesis. By referring to a previous study, at night, *E. densa* consumes oxygen through respiration, leading to DO depletion.

Rodrigues and Thomas (2010) revealed that *E. densa* induces oxygen deficiency from 2.4 to 16.9 mg/L due to dark respiration, concurring with this study. High DO consumption at night created competition with *O. niloticus*, inducing severe hypoxia stress affecting fish metabolism. Duan et al. (2011) suggested that fish in low DO conditions use energy to maintain metabolism, reducing lipid and protein levels. Similarly, Abdel-Tawwab et al. (2015) found that fish in low DO had lower protein and fat levels. Duan et al. (2011) also reported that fish in well-oxygenated water exhibit better feed efficiency and growth performance.

The Effectiveness of *E. densa* as a Natural Oxygenator to Replace Oxygen Pumps in Aquaculture

Environmental hypoxia naturally occurs in aquatic ecosystems when oxygen consumption by plants, bacteria, and animals during the dark cycle exceeds oxygen production or diffusion from the atmosphere (Richard, 2011). Prolonged hypoxia can disrupt respiration, reduce metabolic rates and feed intake, hinder growth, and increase susceptibility to disease (Li et al., 2020). Dissolved oxygen (DO) is therefore critical for freshwater fish, invertebrates, plants, and microbial communities, as it supplies the free oxygen required for survival. Without sufficient DO, most aquatic organisms are unable to endure anoxic or hypoxic conditions for extended periods (Mitchell-Holland et al., 2018).

Growth observations of *Tilapia* sp. demonstrate that fish reared in oxygen-pumped tanks exhibit isometric growth, whereas those in control and *E. densa* tanks show negative allometric growth, highlighting the importance of adequate oxygen supply. Although *E. densa* produces oxygen via photosynthesis, it is unsuitable as a substitute for mechanical oxygenation. This species thrives in diverse aquatic environments due to its ability to utilise bicarbonate as an inorganic carbon source and tolerate low-light conditions; however, it consumes oxygen during the night to aid respiration and growth (Rodrigues & Thomaz, 2010).

Rodrigues and Thomaz (2010) reported substantial oxygen depletion caused by *E. densa* dark respiration, ranging from 2.4 to 16.9 mg/L. These findings support Piria et al. (2022) and contradict Yarrow et al. (2009). Previous studies have presented that DO concentrations typically rise during daylight hours, peak in the afternoon, and fall at night, hitting their lowest levels around early morning (Oldham et al., 2018; Ribaud et al., 2014). In still or lentic water bodies with dense macrophyte coverage, nighttime hypoxia is common as oxygen consumption surpasses replenishment through atmospheric diffusion, particularly near the benthic zone (Ersoy et al., 2020). This indicates that submerged macrophytes are unsuitable as primary oxygen sources in lentic systems.

Although *E. densa* serves as an effective carbon sink, storing carbon at rates comparable to seagrasses and tidal marshes (Drexler et al., 2021), its role as an oxygenator remains limited. Few studies have examined oxygen exchange constraints during the dark respiration

of submerged macrophytes. Mitchell-Holland et al. (2018) reported that *E. densa* is ineffective as an oxygen supplier, whereas *Ceratophyllum demersum* demonstrates greater potential as an oxygenating species. While the utilisation of aquatic plants as biological catalysts for DO enhancement could reduce reliance on energy-intensive oxygen pumps and lower operational and management costs, the present evidence suggests that *E. densa* is unsuitable for this role. Consequently, the aquarium and water garden industries should promote more effective native macrophytes, such as *C. demersum*, to improve water quality and aquatic habitat conditions.

CONCLUSION

Oxygen is crucial for *O. niloticus* as it affects its growth, survival, and feeding. DO levels directly influence the fish's digestion and metabolism. Low oxygen reduces fish quality, resulting in smaller growth, which increases the number of fish needed per kilogram in aquaculture, indirectly impacting agricultural and fishing income. Furthermore, *E. densa* demonstrates marginal effectiveness as a natural oxygenator in aquaculture systems compared to an oxygen pump, as its rate of oxygen production is incompatible with that produced by the pump. Further studies on high dissolved oxygen conditions in aquaculture are required to provide clearer evidence on their effects on cultured fish. Future research should include continuous DO monitoring and better control of confounding factors to isolate hyperoxic effects.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION STATEMENTS

NHR executed and supervised the research, SNK collected data and prepared the manuscript, AZMZ, NAR and SSZ conceived the idea, NAO and SMS handled analysis, and NAY and SNLR edited the manuscript.

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REFERENCES

- Abdel-Tawwab, M., Hagrass, A. E., Elbaghdady, H. A. M., & Monier, M. N. (2014). Dissolved oxygen level and stocking density effects on growth, feed utilisation, physiology, and innate immunity of Nile tilapia, *Oreochromis niloticus*. *Journal of Applied Aquaculture*, 26(4), 340-355. <https://doi.org/10.1080/10454438.2014.959830>

- Abdel-Tawwab, M., Hagra, A. E., Elbaghdady, H. A. M., & Monier, M. N. (2015). Effects of dissolved oxygen and fish size on Nile tilapia, *Oreochromis niloticus* (L.): Growth performance, whole-body composition, and innate immunity. *Aquaculture International*, 23(5), 1261-1274. <https://doi.org/10.1007/s10499-015-9882-y>
- Abu El-Nasr, T. (2021). Mortality of whipfin fish, *Gerres filamentosus* (Cuvier, 1829) in the Red Sea, Egypt. *Bulletin of Faculty of Science*.
- Ahmed, G., Sultana, N., Shamsuddin, M., & Hossain, M. B. (2013). Growth and production performance of monosex tilapia (*Oreochromis niloticus*) fed with homemade feed in earthen mini ponds. *Pakistan Journal of Biological Sciences*, 16(23), 1781-1785. <https://doi.org/10.3923/pjbs.2013.1781.1785>
- Alam, L., Ahmed, M. F., Zolkaply, S. Z. B., & Mokhtar, M. (2017). Risk and benefits of tilapia. In *Tilapia and trout harvesting: Prevalence and benefits* (pp. 1-19). Nova Science Publishers.
- Allen, M. S., & Hightower, J. E. (2010). Fish population dynamics: Mortality, growth, and recruitment. In *Inland fisheries management in North America* (3rd ed., pp. 43-79). American Fisheries Society. <https://doi.org/10.47886/9781934874165>
- Alver, M. O., Føre, M., & Alfredsen, J. A. (2022). Predicting oxygen levels in Atlantic salmon (*Salmo salar*) sea cages. *Aquaculture*, 548, Article 737720. <https://doi.org/10.1016/j.aquaculture.2021.737720>
- Banerjee, A., Chakrabarty, M., Rakshit, N., Bhowmick, A. R., & Ray, S. (2019). Environmental factors as indicators of dissolved oxygen concentration and zooplankton abundance: Deep learning versus traditional regression approach. *Ecological Indicators*, 100, 99-117. <https://doi.org/10.1016/j.ecolind.2018.09.051>
- Bergheim, A., Gausen, M., Næss, A., Hølland, P. M., Krogedal, P., & Crampton, V. (2006). A newly developed oxygen injection system for cage farms. *Aquacultural Engineering*, 34(1), 40-46. <https://doi.org/10.1016/j.aquaeng.2005.04.003>
- Białowiec, A., Sobieraj, K., Pilarski, G., & Manczarski, P. (2019). The oxygen transfer capacity of submerged plant *Elodea densa* in wastewater constructed wetlands. *Water*, 11(3), Article 575. <https://doi.org/10.3390/w11030575>
- Brandt, S. B., Gerken, M., Hartman, K. J., & Demers, E. (2009). Effects of hypoxia on food consumption and growth of juvenile striped bass (*Morone saxatilis*). *Journal of Experimental Marine Biology and Ecology*, 381, S143-S149. <https://doi.org/10.1016/j.jembe.2009.07.028>
- Buentello, J., Gatlin, D. M., & Neill, W. H. (2000). Effects of water temperature and dissolved oxygen on daily feed consumption, feed utilisation, and growth of channel catfish (*Ictalurus punctatus*). *Aquaculture*, 182(3-4), 339-352. [https://doi.org/10.1016/S0044-8486\(99\)00274-4](https://doi.org/10.1016/S0044-8486(99)00274-4)
- Cox, B. (2003). A review of dissolved oxygen modelling techniques for lowland rivers. *The Science of the Total Environment*, 314-316, 303-334. [https://doi.org/10.1016/S0048-9697\(03\)00062-7](https://doi.org/10.1016/S0048-9697(03)00062-7)
- Domenici, P., Steffensen, J. F., & Marras, S. (2017). The effect of hypoxia on fish schooling. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1727), Article 20160236. <https://doi.org/10.1098/rstb.2016.0236>
- Drexler, J. Z., Khanna, S., & Lacy, J. R. (2021). Carbon storage and sediment trapping by *Egeria densa* Planch., a globally invasive, freshwater macrophyte. *The Science of the Total Environment*, 755, Article 142602. <https://doi.org/10.1016/j.scitotenv.2020.142602>

- Duan, Y., Dong, X., Zhang, X., & Miao, Z. (2011). Effects of dissolved oxygen concentration and stocking density on the growth, energy budget and body composition of juvenile Japanese flounder, *Paralichthys olivaceus* (Temminck et Schlegel). *Aquaculture Research*, 42(3), 407-416. <https://doi.org/10.1111/j.1365-2109.2010.02635.x>
- Elvy, J. E., Symonds, J. E., Hilton, Z., Walker, S. P., Tremblay, L. A., Casanovas, P., & Herbert, N. A. (2022). The relationship of feed intake, growth, nutrient retention, and oxygen consumption to feed conversion ratio of farmed saltwater Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture*, 554, Article 738184. <https://doi.org/10.1016/j.aquaculture.2022.738184>
- Ercan, E., Ağralı, N., & Tarkan, A. S. (2015). The effects of salinity, temperature and feed ratio on growth performance of European sea bass (*Dicentrarchus labrax* L., 1758) in the water obtained through reverse osmosis system and a natural river. *Pakistan Journal of Zoology*, 47(3), 625-633. <https://www.cabdirect.org/cabdirect/abstract/20153246981>
- Ersoy, Z., Scharfenberger, U., Baho, D. L., Bucak, T., Feldmann, T., Hejzlar, J., Levi, E. E., Mahdy, A., Nöges, T., Papastergiadou, E., Stefanidis, K., Šorf, M., Søndergaard, M., Trigal, C., Jeppesen, E., & Beklioğlu, M. (2020). Impact of nutrients and water level changes on submerged macrophytes along a temperature gradient: A pan-European mesocosm experiment. *Global Change Biology*, 26(12), 6831-6851. <https://doi.org/10.1111/gcb.15338>
- Febiyanto, F. (2020). Effects of temperature and aeration on the dissolved oxygen (DO) values in freshwater using simple water bath reactor: A brief report. *Walisongo Journal of Chemistry*, 3(1), Article 25. <https://doi.org/10.21580/wjc.v3i1.6108>
- Foss, A., Evensen, T. H., & Øiestad, V. (2002). Effects of hypoxia and hyperoxia on growth and food conversion efficiency in the spotted wolffish *Anarhichas minor* (Olafsen). *Aquaculture Research*, 33(6), 437-444. <https://doi.org/10.1046/j.1365-2109.2002.00693.x>
- Franklin, P. (2014). Dissolved oxygen criteria for freshwater fish in New Zealand: A revised approach. *New Zealand Journal of Marine and Freshwater Research*, 48(1), 112-126. <https://doi.org/10.1080/00288330.2013.827123>
- Gan, L., Liu, Y., Tian, L., Yue, Y., Yang, H., Liu, F., Chen, Y., & Liang, G. (2013). Effects of dissolved oxygen and dietary lysine levels on growth performance, feed conversion ratio and body composition of grass carp, *Ctenopharyngodon idella*. *Aquaculture Nutrition*, 19(6), 860-869. <https://doi.org/10.1111/anu.12030>
- García-Fernández, C., Domínguez-Petit, R., Aldanondo, N., & Saborido-Rey, F. (2020). Seasonal variability of maternal effects in European hake *Merluccius merluccius*. *Marine Ecology Progress Series*, 650, 125-140. <https://doi.org/10.3354/meps13420>
- Gimpel, A., Stelzenmüller, V., Töpsch, S., Galparsoro, I., Gubbins, M., Miller, D., Murillas, A., Murray, A. G., Pınarbaşı, K., Roca, G., & Watret, R. (2018). A GIS-based tool for an integrated assessment of spatial planning trade-offs with aquaculture. *The Science of the Total Environment*, 627, 1644-1655. <https://doi.org/10.1016/j.scitotenv.2018.01.133>
- Goodwin, K., Caraco, N., & Cole, J. (2008). Temporal dynamics of dissolved oxygen in a floating-leaved macrophyte bed. *Freshwater Biology*, 53(8), 1632-1641. <https://doi.org/10.1111/j.1365-2427.2008.01983.x>
- Iskandar, R., & Elrifadah, E. (2013). Growth and feed efficiency of tilapia (*Oreochromis niloticus*) given artificial kiambang feed. *Ziraa'ah Agricultural Scientific Magazine*, 40(1), 18-24.

- Jayraj, P., Machavaram, R., Sahu, G., & Paradkar, V. (2019). Measurement of morphometric dimensions and mechanical properties of rohu fish for design of processing machines. *Journal of Aquatic Food Product Technology*, 28(2), 150-164. <https://doi.org/10.1080/10498850.2019.1569741>
- Kuljanishvili, T., Epitashvili, G., Japoshvili, B., Patoka, J., & Kalous, L. (2021). Finding of Nile tilapia *Oreochromis niloticus* (Cichliformes: Cichlidae) in Georgia, the South Caucasus. *IOP Conference Series: Earth and Environmental Science*, 744(1), Article 012036. <https://doi.org/10.1088/1755-1315/744/1/012036>
- Lakani, F. B., Sattari, M., & Falahatkar, B. (2013). Effect of different oxygen levels on growth performance, stress response and oxygen consumption in two weight groups of great sturgeon *Huso huso*. *Iranian Journal of Fisheries Sciences*, 12(3), 533-549.
- Lara, M. V., Casati, P., & Andreo, C. S. (2002). CO₂-concentrating mechanisms in *Egeria densa*, a submersed aquatic plant. *Physiologia Plantarum*, 115(4), 487-495. <https://doi.org/10.1034/j.1399-3054.2002.1150402.x>
- Li, D., & Liu, S. (2019). Detection of River Water Quality. In: *Water Quality Monitoring and Management: Basis, Technology and Case Studies*, Academic Press, Cambridge, 211-220.
- Li, J., Huang, K., Huang, L., Hua, Y., Yu, K., & Liu, T. (2020). Effects of dissolved oxygen on the growth performance, haematological parameters, antioxidant responses and apoptosis of juvenile GIFT (*Oreochromis niloticus*). *Aquaculture Research*, 51(8), 3079-3090. <https://doi.org/10.1111/are.14684>
- Lugert, V., Thaller, G., Tetens, J., Schulz, C., & Krieter, J. (2016). A review on fish growth calculation: Multiple functions in fish production and their specific application. *Reviews in Aquaculture*, 8(1), 30-42. <https://doi.org/10.1111/raq.12071>
- Mabroke, R. S., Zidan, A. E. F., Tahoun, A., Mola, H. R., State, H. A., & Suloma, A. (2021). Feeding frequency affect feed utilisation of tilapia under biofloc system condition during nursery phase. *Aquaculture Reports*, 19, Article 100625. <https://doi.org/10.1016/j.aqrep.2021.100625>
- Machado, N. R. (2019). *Influence of post hatching age on the sensitivity of zebrafish (Danio rerio) in acute toxicity test* [Doctoral dissertation]. Federal University of Rio Grande do Sul.
- Maharani, H. W., Hasani, Q., Aimm, M. A., Utomo, D. S. C., Santoso, L., Kartini, N., & Kausar, R. A. (2022). Growth performance of tilapia (*Oreochromis niloticus*) cultivated in water from ex-sand pit lakes by phytoremediation treatments. *Journal of Degraded and Mining Lands Management*, 9(2), 3237-3245. <https://doi.org/10.15243/jdmlm.2022.092.3237>
- Mallya, Y. J. (2007). *The effects of dissolved oxygen on fish growth in aquaculture* [Doctoral dissertation]. The United Nations University.
- Mannan, M., Islam, M. S., Rimi, R. H., Suravi, & Meghala, N. T. (2012). Impact of water quality on fish growth and production in semi-intensively managed aquaculture farm. *Bangladesh Journal of Environmental Science*, 23, 108-113.
- McDonald, M. (2012). Measuring lotic and lentic ecosystem diversity in Lake Tamblyn and the McIntyre River to determine water quality. In *Lotic and lentic ecosystem diversity* (pp. 1-15).

- Mengistu, S. B., Mulder, H. A., Benzie, J. A. H., & Komen, H. (2019). A systematic literature review of the major factors causing yield gap by affecting growth, feed conversion ratio and survival in Nile tilapia (*Oreochromis niloticus*). *Reviews in Aquaculture*, 12(2), 524-541. <https://doi.org/10.1111/raq.12331>
- Mitchell-Holland, R., Morris, N. J., & McGregor, P. K. (2018). A comparison of the oxygenating differences of invasive non-native *Lagarosiphon major* and native *Ceratophyllum demersum*. *Bioscience Horizons: The International Journal of Student Research*, 11. <https://doi.org/10.1093/biohorizons/hzy008>
- Mizanur, R. M., Yun, H., Moniruzzaman, M., Ferreira, F., Kim, K., & Bai, S. C. (2014). Effects of feeding rate and water temperature on growth and body composition of juvenile Korean rockfish, *Sebastes schlegeli* (Hilgendorf 1880). *Asian-Australasian Journal of Animal Sciences*, 27(5), 690-699. <https://doi.org/10.5713/ajas.2013.13508>
- Mohamad, S. N., Noordin, W. N. M., Ismail, N. F., & Hamzah, A. (2021). Red hybrid tilapia (*Oreochromis* spp.) broodstock development programme in Malaysia: Status, challenges and prospects for future development. *Asian Fisheries Science*, 34(1). <https://doi.org/10.33997/j.afs.2021.34.1.008>
- Munni, M., Fardus, Z., Mia, M., & Afrin, R. (2013). Assessment of pond water quality for fish culture: A case study of Santosh region in Tangail, Bangladesh. *Journal of Environmental Science and Natural Resources*, 6(2). <https://doi.org/10.3329/jesnr.v6i2.22112>
- Nariswari, S., Yustiati, A., Rostini, I., & Suryadi, I. B. B. (2020). Effect of stocking density on water quality of tilapia (*Oreochromis niloticus* Linnaeus, 1758) in round container with current and venturi aeration system. *World Scientific News*, 145, 397-408.
- Nehemia, A., & Maganira, J. (2012). Length-weight relationship and condition factor of tilapia species grown in marine and fresh water ponds. *Agriculture and Biology Journal of North America*, 3(3), 117-124. <https://doi.org/10.5251/abjna.2012.3.3.117.124>
- Oldham, T., Oppedal, F., & Dempster, T. (2018). Cage size affects dissolved oxygen distribution in salmon aquaculture. *Aquaculture Environment Interactions*, 10, 149-156. <https://doi.org/10.3354/aei00263>
- Piria, M., Radočaj, T., Vilizzi, L., & Britvec, M. (2022). Climate change may exacerbate the risk of invasiveness of non-native aquatic plants: The case of the Pannonian and Mediterranean regions of Croatia. *NeoBiota*, 76, 25-52. <https://doi.org/10.3897/neobiota.76.83320>
- Ribaud, C., Bertrin, V., & Dutartre, A. (2014). Dissolved gas and nutrient dynamics within an *Egeria densa* Planch. bed. *Acta Botanica Gallica*, 161(3), 233-241. <https://doi.org/10.1080/12538078.2014.932703>
- Rifat, S. M., Ashik-E-Rabbani, M., Basir, M. S., & Alam, A. N. (2019). Design and fabrication of an aerator cum oxygen accumulator for live fish transport. *Journal of the Bangladesh Agricultural University*, 17(4), 592-598. <https://doi.org/10.3329/jbau.v17i4.44630>
- Rodrigues, R. B., & Thomaz, S. M. (2010). Photosynthetic and growth responses of *Egeria densa* to photosynthetic active radiation. *Aquatic Botany*, 92(4), 281-284. <https://doi.org/10.1016/j.aquabot.2010.01.009>
- Roslan, M. N. A. M., Estim, A., Maran, B. A. V., & Mustafa, S. (2021). Effects of aquatic plants on nutrient concentration in water and growth performance of fantail goldfish in an aquaculture system. *Sustainability*, 13(20), Article 11236. <https://doi.org/10.3390/su132011236>

- Rouf, M. A., Islam, M. J., Roknuzzaman, M., Siddique, M. N., & Golder, M. R. (2022). Vertical profile of dissolved oxygen and associated water variables in the Pasur-Rupsha estuary of Bangladesh. *Heliyon*, 8(10), Article e10935. <https://doi.org/10.1016/j.heliyon.2022.e10935>
- Saba, A., Ismail, A., Zulkifli, S., Shohaimi, S., Jamil, N., Nawi, N., Ghani, I., Halim, M., & Amal, M. (2020). Checklists, production trends, and potential ecological and socioeconomic impacts of non-native freshwater fishes in Malaysia: A review. *Aquatic Invasions*, 15(4), 646-670. <https://doi.org/10.3391/ai.2020.15.4.07>
- Sangirova, U., Khafizova, Z., Yunusov, I., Rakhmankulova, B., & Kholiyorov, U. (2020). The benefits of development cage fish farming. *E3S Web of Conferences*, 217, Article 09006. <https://doi.org/10.1051/e3sconf/202021709006>
- Saravanan, S., Geurden, I., Figueiredo-Silva, A. C., Kaushik, S. J., Haidar, M. N., Verreth, J. A. J., & Schrama, J. W. (2012). Control of voluntary feed intake in fish: A role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. *British Journal of Nutrition*, 108(8), 1519-1529. <https://doi.org/10.1017/S0007114511006842>
- Schmidt, S., Diallo, I. I., Derriennic, H., Fallou, H., & Lepage, M. (2019). Exploring the susceptibility of turbid estuaries to hypoxia as a prerequisite to designing a pertinent monitoring strategy of dissolved oxygen. *Frontiers in Marine Science*, 6. <https://doi.org/10.3389/fmars.2019.00352>
- Shamso, E., & Hosni, H. (2020). First record of *Egeria densa* Planch. (Hydrocharitaceae) for the aquatic flora of Egypt. *Taeckholmia*, 40(1), 57-63. <https://doi.org/10.21608/taec.2020.24772.1016>
- Sparre, P., Ursin, E., & Venema, S. C. (1989). *Introduction to tropical fish stock assessment. Part 1: Manual*. FAO Fisheries Technical Paper, 3(1), 337. <https://doi.org/10.2331/suisan.79.327>
- Sriyasak, P., Chitmanat, C., Whangchai, N., Promya, J., & Lebel, L. (2015). Effect of water de-stratification on dissolved oxygen and ammonia in tilapia ponds in Northern Thailand. *International Aquatic Research*, 7(4), 287-299. <https://doi.org/10.1007/s40071-015-0113-y>
- Stickney, R. R. (2017). *Aquaculture: An introductory text*. CABI. <https://doi.org/10.1079/9781845935894.0000>
- Tanveer, M., Roy, S. M., Vikneswaran, M., Renganathan, P., & Balasubramanian, S. (2018). Surface aeration systems for application in aquaculture: A review. *International Journal of Fisheries and Aquatic Studies*, 6(5), 342-347.
- Tesfahun, A., & Temesgen, M. (2018). Food and feeding habits of Nile tilapia *Oreochromis niloticus* (L.) in Ethiopian water bodies: A review. *International Journal of Fisheries and Aquatic Studies*, 6(1), 43-47.
- Thorarensen, H., Gústavsson, A., Mallya, Y., Gunnarsson, S., Árnason, J., Arnarson, I., Jónsson, A. F., Smáradóttir, H., Zoega, G. T., & Imsland, A. K. (2010). The effect of oxygen saturation on the growth and feed conversion of Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture*, 309(1-4), 96-102. <https://doi.org/10.1016/j.aquaculture.2010.08.019>
- Tran-Duy, A., Schrama, J. W., Van Dam, A. A., & Verreth, J. A. (2008). Effects of oxygen concentration and body weight on maximum feed intake, growth and hematological parameters of Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, 275(1-4), 152-162. <https://doi.org/10.1016/j.aquaculture.2007.12.024>
- United States Agency for International Development (USAID). (2011). Feed conversion ratio (FCR): How to calculate it and how it is used. *Technical Bulletin*, 1-2.

- Valverde, J. C., López, F. M., & García, B. G. (2006). Oxygen consumption and ventilatory frequency responses to gradual hypoxia in common dentex (*Dentex dentex*): Basis for suitable oxygen level estimations. *Aquaculture*, 256(1-4), 542-551. <https://doi.org/10.1016/j.aquaculture.2006.02.030>
- Yao, Y., Yang, H., & Wang, Y. (2022). Research on the release relationship between dissolved oxygen (DO) and total dissolved gas (TDG) in standing water. *Water Science & Technology Water Supply*, 22(11), 7916-7929. <https://doi.org/10.2166/ws.2022.366>
- Yarrow, M., Marín, V., Finlayson, M., Tironi, A., Delgado, L., & Fischer, F. (2009). The ecology of *Egeria densa* Planchon (Liliopsida: Alismatales): A wetland ecosystem engineer? *Revista Chilena De Historia Natural*, 82(2), 299-313. <https://doi.org/10.4067/S0716-078X2009000200010>
- Yildiz, H. Y., Robaina, L., Pirhonen, J., Mente, E., Domínguez, D., & Parisi, G. (2017). Fish welfare in aquaponic systems: Its relation to water quality with an emphasis on feed and faeces—A review. *Water*, 9(1), Article 13. <https://doi.org/10.3390/w9010013>
- Zhu, W., Wang, L., Dong, Z., Chen, X., Song, F., Liu, N., Yang, H., & Fu, J. (2016). Comparative transcriptome analysis identifies candidate genes related to skin colour differentiation in red tilapia. *Scientific Reports*, 6(1), Article 31347. <https://doi.org/10.1038/srep31347>

Anthocyanin Content, Isoflavone Composition, Antioxidant Activities, and Agronomic Performance of Black Soybean Lines

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ABSTRACT

Modern soybean cultivars should have good agronomic performance in addition to high bioactive properties. This study evaluated anthocyanin content, isoflavone composition, antioxidant activity, and agronomic traits of sixteen black soybean genotypes grown in Mojokerto, East Java, Indonesia. Seed samples were analysed for total anthocyanin content (TAC), antioxidant activity, and isoflavone composition, including daidzein, glycitein, and genistein, in both the seed coat and the cotyledon. Significant variation was observed among genotypes for all biochemical and agronomic traits. TAC was higher in the seed coat than in the seed cotyledon. Genotypes with black seed coats exhibited high TAC in the seed coat, whereas green cotyledon genotypes showed higher TAC in the cotyledon. Isoflavone concentrations ranged from 9.42–27.94 mg/kg for daidzein, 25.18–67.14 mg/kg for glycitein, and 12.93–34.52 mg/kg for genistein. Antioxidant activity in the cotyledon was generally low, while the seed coat exhibited antioxidant activity ranging from low to strong, with three genotypes classified as having strong antioxidant potential. The number of nodes was identified as a useful selection criterion for high-yielding genotypes. The Genotype-by-Trait (GT) biplot showed that the genotype G2 (JPG 15/Anj-884) had the highest values for all isoflavone compositions. Genotypes G6 (Khl/Anj-890), G7 (Khl/Anj-891), and G8 (Khl/Anj-893) performed best in terms

of seed yield and antioxidant activity. Genotypes G9 (Khl/Anj-894), G11 (Khl/Anj-896), and G15 (Mallika) had large seed size and high TAC in both seed and cotyledon. These genotypes have potential for further improvement, either as promising lines or parental lines in developing new black soybean cultivars.

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INTRODUCTION

Black soybean is a promising industrial crop with high nutritional and functional value, such as anthocyanin, isoflavone, and antioxidants (Mitharwal et al., 2024). These functional properties are important for supporting human health. Anthocyanins, which are secondary metabolites and water-soluble pigments found in many fruits, vegetables, and cereal grains, are responsible for the red, purple, or blue colour (Singh et al., 2024). Isoflavones are compounds found in soybeans that act as antioxidants and prevent free radical damage. Similarly, isoflavones from the flavonoid group found in soybean seeds have been shown to benefit people with diabetes mellitus (Kim et al., 2021).

The functional properties of black soybean differ among genotypes, due to variations in genetic, environmental factors, and crop management practices (Choi et al., 2020). A study by Dajanta et al. (2013) found that black soybeans exhibited greater antioxidant properties, total phenolics, and total flavonoids than yellow soybeans. Choi et al. (2020) investigated the relationship between the functional properties and the seed size of 24 black soybeans and found that the large-seeded seeds had higher total anthocyanin and total isoflavone contents than the small seeds, while the small seeds had higher total phenolic content. In another study, it was found that the isoflavone content in the green seed coat and yellow seed coat colours was 2.11 mg/g and 5.24 mg/g, respectively (Bursaca et al. 2017). According to Dhungana et al. (2021), anthocyanins were only found in black soybeans. In contrast to immature soybean seeds, Carrão-Panizzi et al. (2019) found that matured soybean seeds (R8 development stage) have higher levels of isoflavones, anthocyanins, protein, oleic fatty acid, β -glucoside, and aglucone isoflavones. However, it was shown that both anthocyanins and malonyl isoflavones were high in the R7 stage (physiological maturity),

Those research findings offer a potential chance to improve and enhance the functional properties of the soybean genotype. However, the economic value of soybeans, on the other hand, is characterised not only by their nutritional content, but also by commercially valuable agronomic qualities such as seed yield and agronomic traits that maintain high yields (Karges et al., 2022; Ramlal et al., 2023). Several studies on soybeans have shown that seed yield is significantly influenced by yield components, such as the number of pods per plant, the number of seeds per pod, and seed size (Kezar et al., 2023; Tayade et al., 2023; Wang et al., 2025). These traits are frequently used as reliable selection criteria in soybean breeding research and have been shown as significant predictors of seed production (Karyawati & Puspitaningrum, 2021; Mustofa et al., 2021). Furthermore, it has been shown that seed size and seed weight have a significant contribution to yield stability and market value in soybean, especially across different environmental conditions (Egli, 2013). Collectively, these findings emphasise that the integration of bioactive compound evaluation with agronomic performance is essential for the development of superior soybean cultivars.

Several studies on soybeans reported a significant relationship between isoflavones and agronomic traits (Zhang et al., 2014; Kim et al., 2023; Kuswanto et al., 2023). However, a limited study was found on the relationship between antioxidant activity, anthocyanin, and

isoflavone content with agronomic performance. Selecting several soybean genotypes with beneficial functional properties combined with a diversity of agronomic traits, especially seed yield, can help to increase the economic value of soybeans. This study aimed to determine the anthocyanin content, isoflavone composition, and antioxidant activities of black soybean genotypes resulting from crosses of several parental lines, and to examine their relationship with the performance of agronomic traits.

MATERIALS AND METHODS

Plant Genotypes and Study Location

Sixteen black soybean genotypes, consisting of 14 lines derived from crosses and selection among several parental lines and two commercial check cultivars (Mallika and Detam 4), were used in this study (Table 1). Several genotypes listed in Table 1 exhibit brown or blackish-brown seed coats as a result of segregation from crosses between black- and yellow-seeded parents. All genotypes were planted in the experimental field in Mojokerto, East Java, Indonesia, from August to November 2025. The research location has the soil type of Vertisol, C3 climate type based on the Oldeman system, and elevation of 54 a.s.l. The anthocyanin, isoflavone, and antioxidant measurements were performed in the Laboratory of the School of Life Sciences and Technology, Bandung Institute of Technology.

Table 1
The list of soybean genotypes, pedigree, and seed colour

No.	Genotype	Code	Pedigree		Colour	
			Female	Male	Seed Coat	Seed Cotyledon
1	JPG 15/Anj-883	G1	JPG 15	Anjasmoro	Black	Green
2	JPG 15/Anj-884	G2	JPG 15	Anjasmoro	Black	Green
3	JPG 15/Anj-885	G3	JPG 15	Anjasmoro	Black	Green
4	JPG 15/Anj-886	G4	JPG 15	Anjasmoro	Black	Green
5	JPG 15/Anj-887	G5	JPG 15	Anjasmoro	Blackish brown	Green
6	Khl/Anj-890	G6	Khl	Anjasmoro	Blackish brown	Yellow
7	Khl/Anj-891	G7	Khl	Anjasmoro	Blackish brown	Yellow
8	Khl/Anj-893	G8	Khl	Anjasmoro	Black	Yellow
9	Khl/Anj-894	G9	Khl	Anjasmoro	Black	Green
10	Khl/Anj-895	G10	Khl	Anjasmoro	Black	Green
11	Khl/Anj-896	G11	Khl	Anjasmoro	Brown	Green
12	Khl/Anj-898	G12	Khl	Anjasmoro	Brown	Green
13	Khl/Anj-899	G13	Khl	Anjasmoro	Brown	Green
14	Khl/Anj-900	G14	Khl	Anjasmoro	Black	Green
15	Mallika	G15	Local variety		Black	Yellow
16	Detam 4	G16	W9837	G100H	Black	Yellow

Experimental Design and Field Study

The field experiment employed a randomised block design using 16 black soybeans, each of which was replicated four times. The study was conducted in a lowland after rice planting and without any soil tillage. Each genotype was sown in a plot of 2 m × 4.5 m with a planting distance of 40 cm between rows and 15 cm between hills, using two plants per hill. Fertilisers were applied at the time of sowing, consisting of 250 kg/ha Phonska and 100 kg/ha SP-36. Pest, disease, and weed control were managed through regular monitoring. The observations were on the yield and yield components (days to flowering, days to maturity, plant height, number of branches, nodes, filled pods, empty pods, and 100-seed weight).

Determination of Total Anthocyanin Content (TAC)

The seed coat and cotyledon of 16 black soybean genotypes were examined for total anthocyanin content (TAC). Before anthocyanin extraction, the seed coats and cotyledons were manually removed and freeze-dried. After being frozen using liquid nitrogen, 30 grams of each sample were ground with pestles and mortars. The extraction of the TAC followed Giusti and Wrosted (2001). The amount of anthocyanin was quantified by comparing the absorbance to the standard curves at 700 nm and 512 nm at pH 1 and pH 4.5, respectively.

Determination of Antioxidant Activity

The seed coat and cotyledon of black soybeans were examined for antioxidant activity. The DPPH method (Jadid et al. 2017) was used to determine the antioxidant activity. Methanol was used to prepare the DPPH solution, which was subsequently added to extracts at several concentrations (5, 15, 30, 45, and 60 ppm). The absorbance changes were recorded at 517 nm. The standard was ascorbic acid. The following formula was used to calculate the percentage of DPPH inhibition (P_i) as shown in Equation 1:

$$P_i = \frac{(A_b - A_s)}{A_b} \times 100 \quad [1]$$

A_b is the absorbance of the control, A_s is the absorbance of the compound/standard. The antioxidant ability of the sample was expressed as IC_{50} . The antioxidant activity based on the IC_{50} (ppm) was classified according to Molyneux (2004): < 50 ppm indicated very strong antioxidant activity, 50-100 ppm showed strong antioxidant activity, 100-150 ppm denoted intermediate antioxidant activity, and > 150 ppm indicated weak antioxidant activity.

Determination of Isoflavone Composition

Isoflavone content consisting of daidzein, genistein, and glycitein was determined using the methods of Vyn et al. (2002). The concentrations of daidzein, genistein, and glycitein were quantified by comparing sample peak areas with known standards based on calibration curves derived from the standard chromatograms.

Data Analysis

Agronomic traits were analysed using analysis of variance (ANOVA). The relationships between agronomic traits, yield, and bioactive properties were assessed using Pearson correlation in RStudio version 1.3.959 (RStudio Team, 2020). Genotypic performance based on selected agronomic traits and bioactive properties was displayed using a “which-won-where” graph of the genotype-by-trait (GT) biplot (Yan & Rajcan, 2002) using RStudio.

RESULTS AND DISCUSSION

Variation in the Seed Coat and Cotyledon Colour

Soybeans have a variety of seed coat colours, including green, brown, black, and yellow, as well as various cotyledon colours (Gao et al., 2021; Lim et al., 2021). Soybeans with black seed coats can be divided into two classes based on the colour of their cotyledons, which can be green or yellow (Jo et al., 2021). In this study, the 16 black soybeans consisted of 10 genotypes with black seed coat colours, three genotypes had blackish brown colours, and the rest (three genotypes) had brown seed coat colours. The cotyledon consisted of the green cotyledon (11 genotypes) and the yellow cotyledon (five genotypes) (Figure 1). The formation of a black seed coat is mainly attributed to the accumulation of anthocyanins and is genetically regulated by the *i* allele. The most common anthocyanins in black-seeded soybean types are delphinidin 3-O-glucoside, petunidin 3-O-glucoside, and cyanidin 3-O-glucoside (Choung et al., 2001).

The variation in the seed coat colour of black soybean could be due to gene action. Gene *R* is responsible for seed hilum colour variation, while gene *T* is responsible for brown pubescent colour. The allelic combination *RT* displays a black seed coat colour, while the allelic combination *rT* displays a brown seed coat colour (Bernard & Weiss, 1973). In Indonesia, information on the genetic variation of the seed coat and cotyledon colours of black soybean is still limited. Meanwhile, in South Korea, soybeans with black seed coats and green cotyledons exhibit narrow genetic variation (Jo et al., 2021).



Figure 1. Variation in seed coat and cotyledon colours of 16 black soybean genotypes (A = seed colour; B = cotyledon colour; C = seed coat colour)

Anthocyanin Content

Black soybeans have the potential to provide nutritional and health benefits for humans (Bhartiya et al., 2020) due to the noticeable variations in compounds compared to yellow soybeans (Anjum et al., 2022; Li et al., 2024), such as anthocyanin. In this study, the TAC was measured in both the seed coat and the cotyledon. The average TAC of the black soybean genotypes was higher in the seed coat (283.69 mg/100 g) than in the cotyledon (118.25 mg/100 g). Previous studies also found a high amount of anthocyanin in the seed coat (Choi et al., 2020; Zhang et al., 2011). It has been estimated that over 99% of the total anthocyanin in black soybeans is found in the epidermal palisade layer of the seed coat, which is primarily responsible for the black colouration (Zhang et al., 2011).

The TAC range in the seed coat was 54.98-734.51 mg/100 g (Figure 2a) while it was 13.47-272.40 mg/100 g in the cotyledon (Figure 2b). The highest anthocyanin content in the seed coat was Khl/Anj-894 (734.51 mg/100 g), higher than that of the check cultivar of Mallika (707.29 mg/100 g). Both genotypes had black seed coat colour. The highest anthocyanin content in seed cotyledons was Khl/Anj-896 (272.40 mg/100 g) and Khl/Anj-900 (229.02 mg/100 g)—both had green cotyledon colour. Soybeans with black seed coats and green cotyledons have been commonly utilised in medicinal treatments in Japan, Korea, and China (Jo et al., 2021). A study on Korean black soybean revealed that the range of TAC was from 19.8 to 1,420.4 mg/100 g (Lee et al., 2016). Another study conducted by Choi et al. (2020) linked anthocyanin content with soybean seed size and reported that the TAC varied in small seeds (280.16-1085.89 mg/100 g), medium seeds (189.461-1191.028 mg/100 g), and large seeds (457.508-2633.454 mg/100 g). The available information on the variation and anthocyanin content among soybean genotypes provides an excellent opportunity to develop soybean varieties with high anthocyanin content.

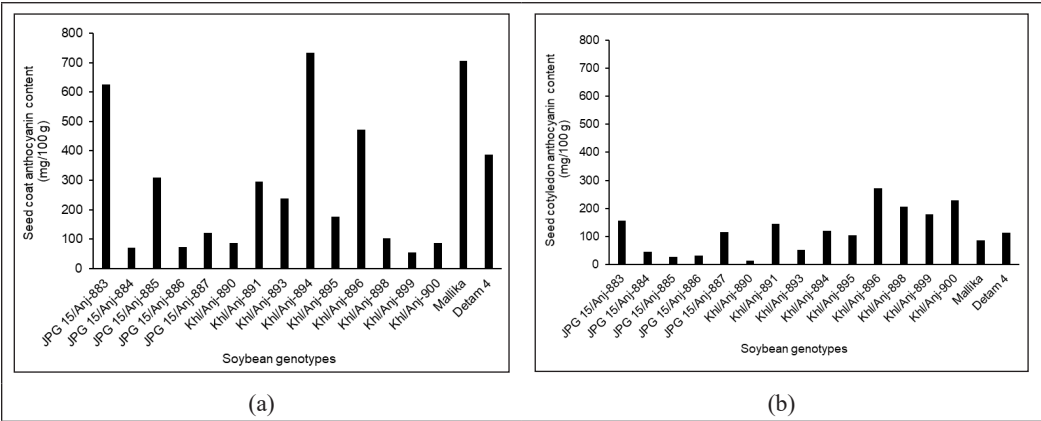


Figure 2. Anthocyanin content of 16 black soybean genotypes in the (a) seed coat and (b) seed cotyledon

Isoflavone Composition

The isoflavone composition, which consisted of daidzein, glycitein, and genistein, varied among black soybean genotypes. The contents of daidzein, glycitein, and genistein ranged from 9.42 to 27.94 mg/kg, 26.80 to 67.14 mg/kg, and 12.93 to 34.52 mg/kg, respectively. Among 16 genotypes, the average glycitein was higher than daidzein and genistein. Several studies found a higher variation in daidzein than in genistein (Anjum et al., 2022; Sumardi et al., 2017). Furthermore, another study found a higher genistein content (de Oliveira et al. 2024). These results suggest that the variation in the isoflavone content was influenced by environment (E), genotype (G), and the GE interaction (Choi et al., 2020).

The check cultivars of Mallika and Detam 4 had the highest daidzein (27.94 and 25.39 mg/kg, respectively). The Detam 4 had the highest glycitein (67.14 mg/kg), followed by Khl/Anj-896 (49.30 mg/kg). The highest genistein was found in JPG 15/Anj-883 and Mallika n (34.52 and 33.66 mg/kg, respectively). These findings show that Mallika has excellent daidzein and genistein contents, while Detam 4 has excellent daidzein and glycitein contents. The JPG 15/Anj-883 showed high glycitein and genistein (Figure 3). The biological activities of genistein and daidzein are widely recognised. However, genistein was reported to have about ten times higher biological activity than daidzein and glycitein (Kocar et al., 2019). Numerous significant effects of genistein have been reported, including anti-cancer activity (Yu et al., 2020), suppression of axillary osmidrosis (Saito et al., 2020), and potential nematocidal activities against soybean cyst nematodes (Ma et al., 2022). Several studies have also shown that genistein and other isoflavones derived from soybean roots affect the diversity of microbial development, notably fungus populations (Colpas et al., 2003; Guo et al., 2011).

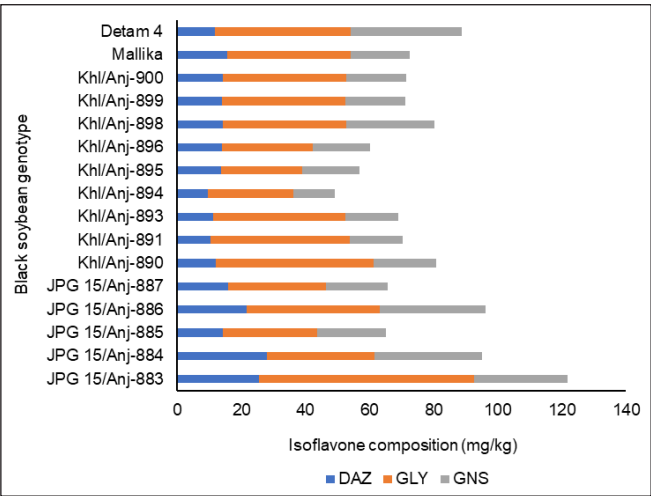


Figure 3. Isoflavone composition (daidzein, glycitein, and genistein) of 16 black soybean genotypes

Antioxidant Activity

Antioxidants are compounds that support human health by neutralising damaging free radicals in the body. These substances help to minimise oxidative stress, which is linked to a variety of chronic diseases and the ageing process. Previous research has revealed that the extract of black soybean showed strong antioxidant activity, indicating its potential use as an alternative anti-ageing agent due to the presence of phenolic chemicals that work as antioxidants (Asan et al., 2019). In this study, the determination of antioxidant activity was performed in the seed coat and cotyledon. The IC₅₀ value was used to measure the antioxidant activity. It is determined as the substrate concentration required that causes a 50% loss of the DPPH activity (Molyneux, 2004). Hence, the lower the IC₅₀ value, the higher the antioxidant activity (Rivero-Cruz et al., 2020). Accordingly, the black soybean cotyledon of all genotypes has a weak antioxidant activity, with the IC₅₀ value ranging from 4,115.47 - 6,813.84 ppm. In contrast, the antioxidant activity in the seed coat ranged from strong to weak, with a range of 5,595.05 -78.29 ppm. These findings are consistent with previous studies, which reported that antioxidant activity was primarily found in the seed coat (Soedarjo, 2019; Lim et al., 2021).

In this study, black soybean lines (KhI/Anj-894, KhI/Anj-896, and KhI/Anj-898) showed strong antioxidant activity in the seed coat. Meanwhile, eight lines exhibited intermediate antioxidant activity, while the other lines had low antioxidant activity (Table 2). Three lines with strong antioxidant activity had black and brown seed coats. Significant polymerised procyanidin and anthocyanin content in the black seed coat of soybeans may explain the excellent antioxidant effect (Takahata et al., 2001). In line with research, Lee et al. (2020) found that soybean genotype 42 KSBLs exhibited higher levels of phytochemicals and antioxidant activities.

A study by Prvulović et al. (2016) showed variation in the antioxidant activity of five soybean genotypes. In other crops, such as *Vigna unguiculata*, there was also variation in antioxidant potential (Saeed et al., 2020). A high variation in the antioxidant activity of black soybean (10.99-20.38 µmol TE/g) was also reported by Yusnawan et al. (2016).

Table 2
The IC₅₀ values and antioxidant activities of 16 black soybean genotypes

No.	Genotype	Cotyledon		Seed coat	
		IC ₅₀ value	Category	IC ₅₀ value	Category
1	JPG 15/Anj-883	4,606.23	Weak	116.23	Intermediate
2	JPG 15/Anj-884	4,115.47	Weak	129.85	Intermediate
3	JPG 15/Anj-885	4,876.50	Weak	164.70	Intermediate
4	JPG 15/Anj-886	4,701.73	Weak	114.72	Intermediate
5	JPG 15/Anj-887	5,212.34	Weak	105.26	Intermediate
6	Khl/Anj-890	6,782.94	Weak	4,127.60	Weak
7	Khl/Anj-891	5,115.20	Weak	5,595.05	Weak
8	Khl/Anj-893	5,928.30	Weak	4,381.18	Weak
9	Khl/Anj-894	4,273.08	Weak	78.29	Strong
10	Khl/Anj-895	4,479.00	Weak	104.89	Intermediate
11	Khl/Anj-896	4,989.82	Weak	82.77	Strong
12	Khl/Anj-898	5,312.81	Weak	84.09	Strong
13	Khl/Anj-899	4,365.55	Weak	3,292.59	Weak
14	Khl/Anj-900	4,875.08	Weak	2,892.42	Weak
15	Malika	6,813.84	Weak	130.07	Intermediate
16	Detam 4	4,833.56	Weak	104.89	Intermediate

Performance and Correlation of Agronomic Traits

The agronomic traits showed variation among the black soybean genotypes (Table 3). The performance of the agronomic traits was presented in Figure 4. Based on the performance of the agronomic traits, it was noted that the plants grew normally. The range of seed was 2.54 to 3.89 t/ha, with a range of days to maturity from 81 to 87 days. These indicate that soybean genotypes have a high genetic potential.

Genotype and environment have a significant impact on soybean seed yield and yield components. As new cultivars are developed through breeding, it is important for breeders to investigate the correlations between yield and yield components (Mecha et al., 2017). The study of the effect of agronomic traits on seed yield showed that plant height (r = -0.602*), number of nodes (r = 0.502*), and number of empty pods (r = -0.703*) had a significant correlation with seed yield (Figure 5).

Table 3
Analysis of variance for agronomic traits of 16 black soybean genotypes

Traits	Symbol	Mean Square	
		Replication	Genotype
Days to flowering (days)	DTF	0.5208 ^{ns}	3.2291 ^{**}
Days to maturity (days)	DTM	7.8541 ^{**}	19.0958 ^{**}
Plant height (cm)	PHG	694.9980 ^{**}	192.8692 ^{**}
Number of branches	NOB	0.1297 ^{ns}	3.3707 ^{**}
Number of nodes	NON	54.1696 ^{ns}	118.2683 ^{**}
Number of filled pods	NFP	388.0618 ^{**}	322.8526 ^{**}
Number of empty pods	NEP	2.4340 ^{ns}	4.6653 ^{**}
100 seed weight (g)	SWG	0.3536 [*]	4.7384 ^{**}
Seed yield (t/ha)	YIELD	0.0858 ^{**}	0.4085 ^{**}

Note. ns = not significant; * = significant at 5 % probability level ($p < 0.05$); ** = significant at 1 % probability level ($p < 0.01$)

In this study, plant height was negatively correlated with seed yield. A higher plant may not consistently demonstrate a higher yield because tall plants may have a denser canopy, which limits the interception of photosynthetically active radiation in the plant's lower parts (Vu et al., 2019). Higher seed yields have been observed in soybean cultivars with greater efficiency in intercepting photosynthetically active radiation within the vegetative canopy (Müller et al., 2017).

In this study, the number of nodes showed a positive correlation with seed yield. This result is similar to previous studies (Hapsari et al., 2021; Karyawati & Puspitaningrum, 2021). This finding implies that the number of filled pods was influenced by the number of nodes. The number of pods per unit area is frequently correlated with seed yield, suggesting that the number of pods with nodes affects soybean yield (Egli, 2013). Additionally, the number of nodes per plant could contribute to the changes in the number of pods per plant (Egli, 2013; Wei & Molin, 2020). The present study also revealed a significant negative correlation between the number of empty pods and seed yield, indicating that an increase in non-reproductive nodes may reduce seed yield potential (Du et al., 2020; Egli, 2013). Information on the relationship between soybean yield and yield components can be beneficial in the selection of parental lines for producing cultivars with higher genetic yield potential. Based on these findings, the indirect selection for short plants could increase the seed yield in soybean, and the number of nodes can be used as one of the indicators to obtain high-yielding soybean genotypes in the breeding selection stage.

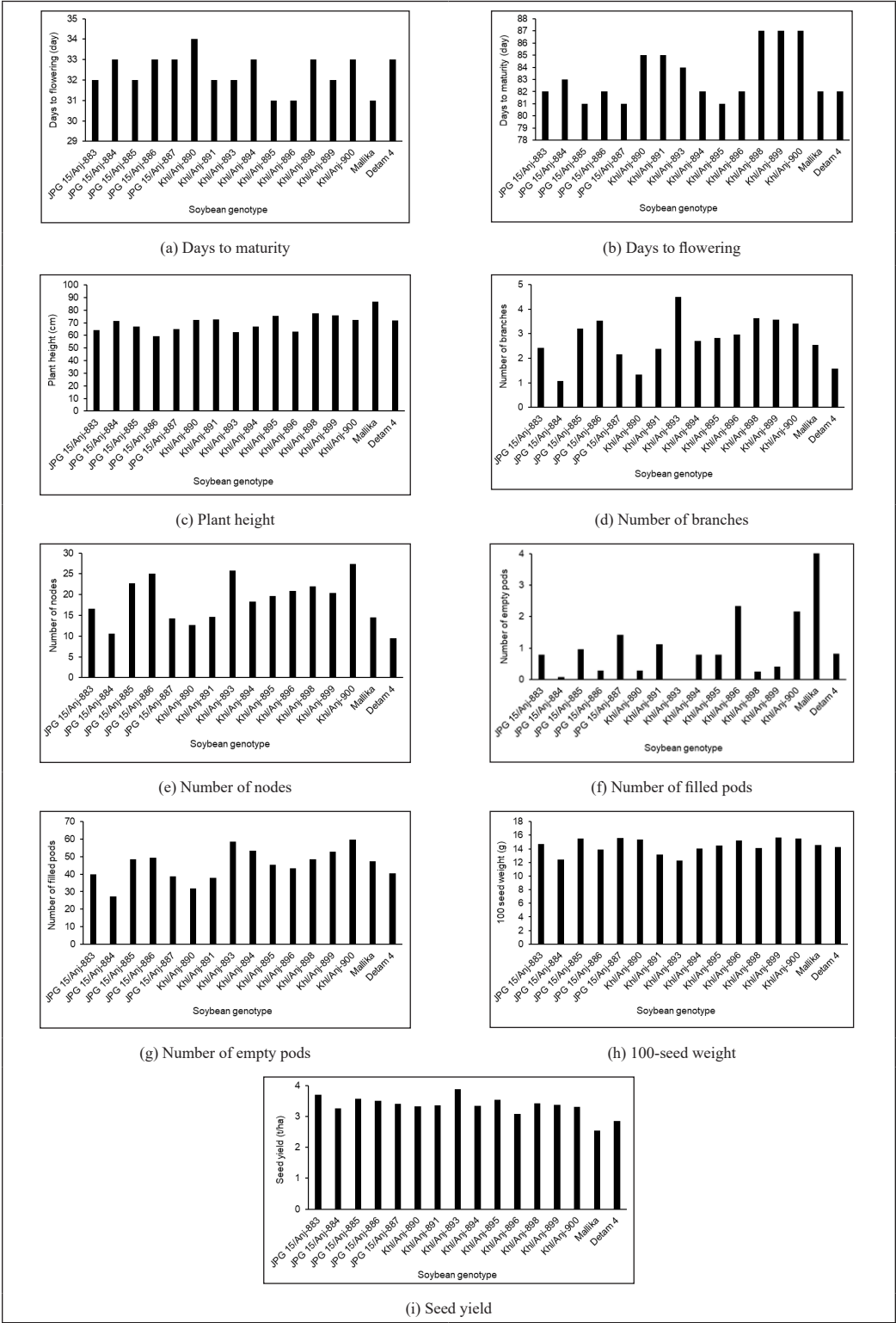


Figure 4. The agronomic trait performance of 16 black soybean genotypes

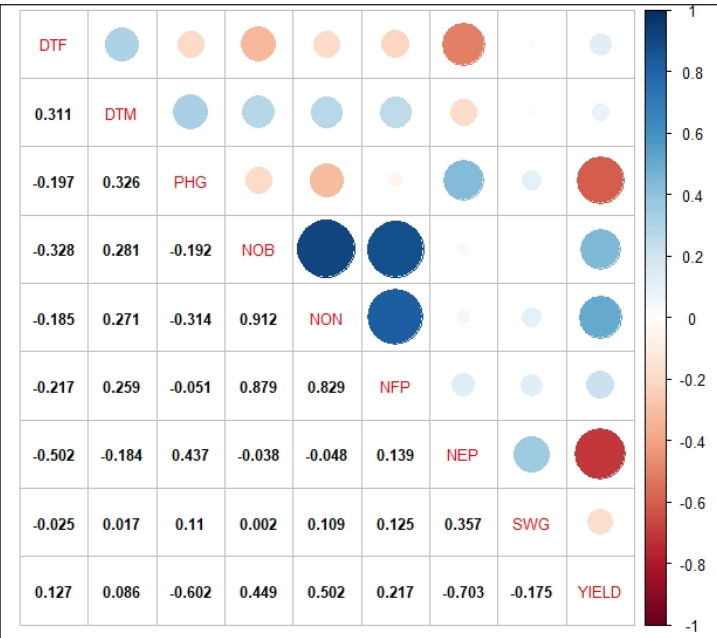


Figure 5. Correlation among agronomic traits of 16 black soybean genotypes. The limit of significance correlation is $\geq \pm 0.50$; the agronomic trait codes are shown in Table 3

Correlation between Yield and Bioactive Properties

No significant correlation was found between seed yield and the bioactive properties (TAC, antioxidant activities, isoflavone), nor among the bioactive properties, except for daidzein with genistein (Figure 6). A non-significant correlation between seed yield and both daidzein and genistein was also reported by Laurenz et al. (2017). However, a previous study by Vyn et al. (2002) revealed a positive correlation between isoflavone concentrations and seed yield. In the present study, daidzein and genistein were strongly and positively correlated with each other ($r = 0.676^{**}$).

The individual isoflavones (daidzein, glycitein, genistein) also showed no significant correlation with the TAC and antioxidant activity, respectively. This finding is in line with earlier studies, which reported no significant correlation between anthocyanins and isoflavones, or between antioxidant activity and isoflavone content (Choi et al., 2020; Lee et al., 2016). Another study found negative or weak associations between individual isoflavones with particular anthocyanins, indicating a trade-off between their biosynthesis pathways (Wu et al., 2017). However, the lack of significant correlation between the seed yield and the functional properties found in this study indicates the possibility of improving these functional properties without significantly lowering seed yield.



Figure 6. Correlation between yield and functional properties of 16 black soybean genotypes. The limit of significance correlation is $\geq \pm 0.50$; DAZ = daidzein; GLY = glycitein; GNS = genistein; TACs = total anthocyanin content in seed; TACsc = total anthocyanin content in seed cotyledon; ANTsc = antioxidant activity in seed; YLD = seed yield

Genotype Evaluation Based on Selected Agronomic Traits and Bioactive Properties

The characteristics of modern soybean varieties are determined by bioactive properties, yield potential, and agronomic performance. Large seed size is one of these characteristics that could be improved to meet the specific demand of society. In addition, large-seeded soybeans were used by the industry as raw material for tempeh production (Krisnawati & Adie, 2015). In this study, the genotype evaluation was based on important agronomic traits (large seed size and high yield) and bioactive properties.

A GT biplot with a polygon view was used to help identify genotypes with the highest values for one or more traits (Figure 7). The biplot was divided into six sectors, with ten traits falling into three sectors. The genotype with the greatest value of the traits within each sector was considered to be the vertex genotype (Paramesh et al., 2016). Figure 7 illustrates that the vertex genotypes are G2, G6, G7, G8, G9, G11, and G15. The GT biplot suggests that G2 has the highest or near-highest value for all isoflavones (daidzein, glycitein, genistein). The genotypes G6, G7, and G8 demonstrated superior performance in seed yield and antioxidant activity in the seed and cotyledon. The genotypes G9, G11, and G15 exhibited superior performance in the large seed size and TAC in the seed and seed cotyledon.

The identified soybean genotypes with specific superior traits could be used as elite genetic resources for soybean breeding programs or be further developed to improve black soybean cultivars for industrial purposes. For example, genotypes with high daidzein, glycitein, and genistein contents could be utilised as parents to breed soybean lines with enhanced isoflavone levels and composition, which are desirable by the industry.

Previous research findings have focused on evaluating the content of bioactive properties. However, research that simultaneously assesses both yield-related agronomic traits and functional properties remains limited. By applying the GT biplot approach, the results of this study provide valuable insights for identifying modern black soybean varieties that combine excellent agronomic performance with high health benefits.

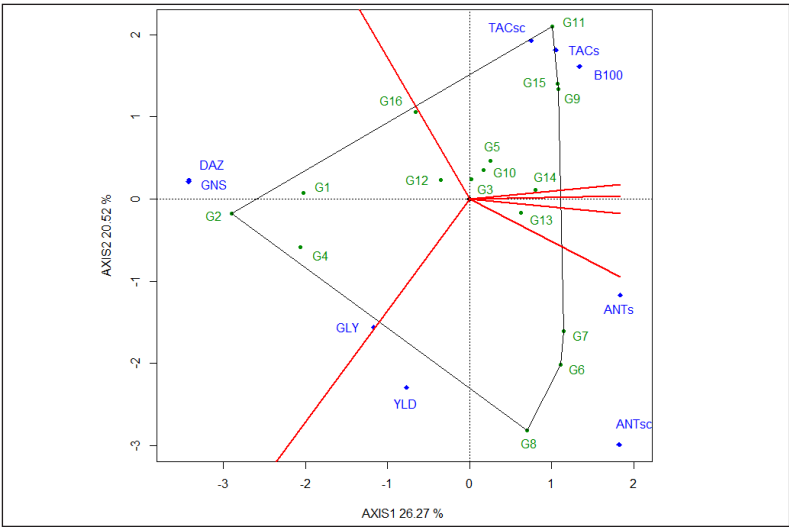


Figure 7. Polygon view of the “which-won-where” graph of genotype-by-trait biplot, illustrating which genotypes exhibit the highest values for specific traits. DAZ = daidzein; GLY = glycitein; GNS = genistein; TACs = total anthocyanin content in seed; TACsc = total anthocyanin content in seed cotyledon; ANTsc = antioxidant activity in seed; ANT = antioxidant activity in seed cotyledon; B100 = 100-seed weight; YLD = seed yield

CONCLUSION

The anthocyanin content, isoflavone composition, antioxidant activity, and agronomic traits of black soybeans varied among genotypes. The anthocyanin content of black soybeans in the seed coat was higher than in the seed cotyledon. The isoflavone composition showed that the average glycitein content was higher than daidzein and genistein. The antioxidant activity in the seed coat ranged from low to strong, while the seed cotyledon showed a moderate to strong antioxidant activity. The number of nodes can be used as one of the selection indicators to obtain high-yielding soybean genotypes.

The soybean genotype G2 (JPG 15/Anj-884) had the highest values for the traits of all isoflavones (daidzein, glycitein, genistein). Genotypes G6 (Khl/Anj-890), G7 (Khl/Anj-891), and G8 (Khl/Anj-893) exhibited superior performance in seed yield and antioxidant activity in both seed and cotyledon. Genotypes G9 (Khl/Anj-894), G11 (Khl/Anj-896), and G15 (Mallika) showed superior performance in seed size and total anthocyanin content in both the seed and the cotyledon. These genotypes represent promising candidates for further improvement of new black soybean cultivars, either through selection as pure lines or as parental lines in the breeding program.

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REFERENCES

- Anjum, S., Rana, S., Dasila, K., Agnihotri, V., Pandey, A., & Pande, V. (2022). Comparative nutritional and antimicrobial analysis of Himalayan black and yellow soybean and their okara. *Journal of the Science of Food and Agriculture*, 102(12), 5358-5367. <https://doi.org/10.1002/jsfa.11889>
- Asan, T., Lister, I. N. E., Fachrial, E., Amalia, A., Widowati, W., Samin, B., & Liena, L. (2019). Potency of black soybean (*Glycine max* (L.) Merr) extract and daidzein as antioxidant and antihyaluronidase. *Traditional Medicine Journal*, 24(1), 52-58. <https://doi.org/10.22146/mot.43615>
- Bernard, R. L., & Weiss, M. G. (1973). Qualitative genetics of soybean. In B. E. Caldwell (Ed.), *Soybeans: Improvement, production, and uses* (pp. 117-154). The American Society of Agronomy.
- Bhartiya, A., Aditya, J. P., Pal, R. S., Chandra, N., Kant, L., & Pattanayak, A. (2020). Bhat (black soybean): A traditional legume with high nutritional and nutraceutical properties from NW Himalayan region of India. *Indian Journal of Traditional Knowledge*, 19(2), 307-319. <https://doi.org/10.56042/ijtk.v19i2.35346>
- Bursaca, M., Krstonosic, M. A., Miladinović, J., Malenčić, D., Gvozdenović, L., & Cvejić, J. H. (2017). Isoflavone composition, total phenolic content and antioxidant capacity of soybeans with colored seed coat. *Natural Product Communications*, 12(4), 527-532. <https://doi.org/10.1177/1934578X1701200417>
- Carrão-Panizzi, M. C., Silva, B. dos S., Leite, R. S., Godoy, R. L. de O., Santiago, M. C. P. de A., Felberg, I., & Oliveira, M. C. N. (2019). Isoflavone, anthocyanin, and fatty acid contents of vegetable-type soybean grains at different maturity stages. *Pesquisa Agropecuária Brasileira*, 54, Article e00032. <https://doi.org/10.1590/s1678-3921.pab2019.v54.00032>

- Choi, Y. M., Yoon, H., Lee, S., Ko, H. C., Shin, M. J., Lee, M. C., Hur, O. S., Ro, N. Y., & Desta, K. T. (2020). Isoflavones, anthocyanins, phenolic content, and antioxidant activities of black soybeans (*Glycine max* (L.) Merrill) as affected by seed weight. *Scientific Reports*, 10, Article 19960. <https://doi.org/10.1038/s41598-020-76985-4>
- Choung, M. G., Baek, I. Y., Kang, S. T., Han, W. Y., Shin, D. C., Moon, H. P., & Kang, K. H. (2001). Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). *Journal of Agricultural and Food Chemistry*, 49(12), 5848-5851. <https://doi.org/10.1021/jf010550w>
- Colpas, F. T., Ono, E. O., Rodrigues, J. D., & de Souza Passos, J. R. (2003). Effects of some phenolic compounds on soybean seed germination and on seed-borne fungi. *Brazilian Archives of Biology and Technology*, 46(2), 155-161. <https://doi.org/10.1590/S1516-89132003000200003>
- Dajanta, K., Janpum, P., & Leksing, W. (2013). Antioxidant capacities, total phenolics and flavonoids in black and yellow soybeans fermented by *Bacillus subtilis*: A comparative study of Thai fermented soybeans (thua nao). *International Food Research Journal*, 20(6), 3125-3132.
- de Oliveira, I. C., Santana, D. C., de Oliveira, J. L. G., Silva, E. V. M., Seron, A. C. S. D., Blanco, M., Teodoro, L. P. R., Junior, C. A. S., Baio, F. H. R., Alves, C. Z., & Teodoro, P. E. (2024). Flavonoids and their relationship with the physiological quality of seeds from different soybean genotypes. *Scientific Reports*, 14, Article 17008. <https://doi.org/10.1038/s41598-024-68117-z>
- Dhungana, S. K., Seo, J. H., Kang, B. K., Park, J. H., Kim, J. H., Sung, J. S., Baek, I. Y., Shin, S. O., & Jung, C. S. (2021). Protein, amino acid, oil, fatty acid, sugar, anthocyanin, isoflavone, lutein, and antioxidant variations in coloured seed-coated soybeans. *Plants*, 10(9), Article 1765. <https://doi.org/10.3390/plants10091765>
- Du, Y., Zhao, Q., Li, S., Yao, X., Xie, F., & Zhao, M. (2020). Shoot/root interactions affect soybean photosynthetic traits and yield formation: A case study of grafting with record-yield cultivars. *Frontiers in Plant Science*, 10, Article 445. <https://doi.org/10.3389/fpls.2019.00445>
- Egli, D. (2013). The relationship between the number of nodes and pods in soybean communities. *Crop Science*, 53(4), 1668-1676. <https://doi.org/10.2135/cropsci2012.11.0663>
- Gao, R., Han, T., Xun, H., Zeng, X., Li, P., Li, Y., Wang, Y., Shao, Y., Cheng, X., Feng, X., Zhao, J., Wang, L., & Gao, X. (2021). MYB transcription factors GmMYBA2 and GmMYBR function in a feedback loop to control pigmentation of seed coat in soybean. *Journal of Experimental Botany*, 72(12), 4401-4418. <https://doi.org/10.1093/jxb/erab152>
- Giusti, M. M., & Wrosted, R. E. (2001). Characterisation and measurement of anthocyanin by UV-Visible spectroscopy. In R. E. Wrosted, E. A. Acree, M. H. Dekker, D. S. Penner, S. J. Reid, C. F. Schwan, D. Shoemaker, & P. S. Smith (Eds.), *Handbook of food analytical chemistry: Pigments, colourants, flavours, texture, and bioactive food components*. John Wiley & Sons. <https://doi.org/10.1002/0471142913.faf0102s00>
- Guo, Z. Y., Kong, C. H., Wang, J. G., & Wang, Y. F. (2011). Rhizosphere isoflavones (daidzein and genistein) levels and their relation to the microbial community structure of mono-cropped soybean soil in field and controlled conditions. *Soil Biology and Biochemistry*, 43(11), 2257-2264. <https://doi.org/10.1016/j.soilbio.2011.07.022>

- Hapsari, R. T., Adie, M. M., & Krisnawati, A. (2021). Yield performance and agronomic character association in soybean genotypes. *IOP Conference Series: Earth and Environmental Science*, 911(1), Article 012023. <https://doi.org/10.1088/1755-1315/911/1/012023>
- Jadid, N., Hidayati, D., Hartanti, S.R., Arraniry, B.A., Rachman, R.Y., & Wikanta, W. (2017). Antioxidant activities of different solvent extracts of *Piper retrofractum* Vahl. using DPPH assay. *AIP Conference Proceedings*, 1854, Article 020019. <https://doi.org/10.1063/1.4985410>
- Jo, H., Lee, J.Y., Cho, H., Choi, H.J., Son, C.K., Bae, J.S., Bilyeu, K., Song, J.T., & Lee, J.D. (2021). Genetic diversity of soybeans (*Glycine max* (L.) Merr.) with black seed coats and green cotyledons in Korean germplasm. *Agronomy*, 11(3), Article 581. <https://doi.org/10.3390/agronomy11030581>
- Karges, K., Bellingrath-Kimura, S. D., Watson, C. A., Stoddard, F. L., Halwani, M., & Reckling, M. (2022). Agro-economic prospects for expanding soybean production beyond its current northerly limit in Europe. *European Journal of Agronomy*, 133, Article 126415. <https://doi.org/10.1016/j.eja.2021.126415>
- Karyawati, A. S., & Puspitaningrum, E. S. V. (2021). Correlation and path analysis for agronomic traits contributing to yield in 30 genotypes of soybean. *Biodiversitas*, 22(3), 1146-1151. <https://doi.org/10.13057/biodiv/d220309>
- Kezar, S., Ballagh, A., Kankarla, V., Sharma, S., Sharry, R., & Lofton, J. (2023). Response of soybean yield and certain growth parameters to simulated reproductive structure removal. *Agronomy*, 13(3), Article 927. <https://doi.org/10.3390/agronomy13030927>
- Kim, I. S., Kim, C. H., & Yang, W. S. (2021). Physiologically active molecules and functional properties of soybeans in human health—A current perspective. *International Journal of Molecular Sciences*, 22(8), Article 4054. <https://doi.org/10.3390/ijms22084054>
- Kim, J. M., Seo, J. S., Lee, J. W., Lyu, J. I., Ryu, J., Eom, S. H., Ha, B. K., & Kwon, S. J. (2023). QTL mapping reveals key factors related to the isoflavone contents and agronomic traits of soybean (*Glycine max*). *BMC Plant Biology*, 23(1), Article 517. <https://doi.org/10.1186/s12870-023-04519-x>
- Kocar, M. M., Vila, S., Petrovic, S., Rebekic, A., Sudaric, A., Duvnjak, T., Kulundzic, A. K., & Josipovic, A. (2019). Isoflavone concentrations in soybeans suitable for growing in Europe. *Genetika*, 51(1), 47-59. <https://doi.org/10.2298/GENSR1901047M>
- Krisnawati, A., & Adie, M. M. (2015). Selection of soybean genotypes by seed size and its prospects for industrial raw material in Indonesia. *Procedia Food Science*, 3, 355-363. <https://doi.org/10.1016/j.profoo.2015.01.039>
- Kuswanto, H., Ginting, E., Yusnawan, E., Utomo, J. S., & Sundari, T. (2023). Agronomic performance, seed chemical composition, and bioactive components of selected Indonesian soybean genotypes (*Glycine max* [L.] Merr.). *Open Agriculture*, 8(1), Article 20220229. <https://doi.org/10.1515/opag-2022-0229>
- Laurenz, R., Tumbalam, P., Naeve, S., & Thelen, K. D. (2017). Determination of isoflavone (genistein and daidzein) concentration of soybean seed as affected by environment and management inputs. *Journal of the Science of Food and Agriculture*, 97(10), 3342-3347. <https://doi.org/10.1002/jsfa.8184>
- Lee, K. J., Baek, D., Lee, G., Cho, G., So, Y., Lee, J., Ma, K., Chung, J., & Hyun, D. J. (2020). Phytochemicals and antioxidant activity of Korean black soybean (*Glycine max* L.) landraces. *Antioxidants*, 9(3), Article 213. <https://doi.org/10.3390/antiox9030213>

- Lee, M., Sorn, S. R., Park, Y., & Park, H. (2016). Anthocyanin rich-black soybean testa improved visceral fat and plasma lipid profiles in overweight/obese Korean adults: A randomised controlled trial. *Journal of Medicinal Food*, 19(11), 995-1003. <https://doi.org/10.1089/jmf.2016.3762>
- Li, S., Chen, J., Hao, X., Ji, X., Zhu, Y., Chen, X., & Yao, Y. (2024). A systematic review of black soybean (*Glycine max* (L.) Merr.): Nutritional composition, bioactive compounds, health benefits, and processing to application. *Food Frontiers*, 5(3), 1188-1211. <https://doi.org/10.1002/fft2.376>
- Lim, Y. J., Kwon, S. J., Qu, S., Kim, D. G., & Eom, S. H. (2021). Antioxidant contributors in seed, seed coat, and cotyledon of γ -ray-induced soybean mutant lines with different seed coat colours. *Antioxidants*, 10(3), Article 353. <https://doi.org/10.3390/antiox10030353>
- Ma, Y., Yuan, R., Sikandar, A., Zhu, X., Duan, Y., & Wang, Y. (2022). Genistein and daidzein effects on the physiological indices of soybean cyst nematode. *Scientia Agricola*, 79(2), 1-8. <https://doi.org/10.1590/1678-992x-2020-0298>
- Mecha, B., Alamerew, S., Assefa, A., Assefa, E., & Dutamo, D. (2017). Correlation and path coefficient studies of yield and yield associated traits in bread wheat (*Triticum aestivum* L.) genotypes. *Advances in Plants and Agriculture Research*, 6(5), 128-136. <https://doi.org/10.15406/apar.2017.06.00226>
- Mitharwal, S., Saini, A., Chauhan, K., Taneja, N. K., & Oberoi, H. S. (2024). Unveiling the nutrient-wealth of black soybean: A holistic review of its bioactive compounds and health implications. *Comprehensive Reviews in Food Science and Food Safety*, 23(5), Article e70001. <https://doi.org/10.1111/1541-4337.70001>
- Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology*, 26(2), 211-219.
- Müller, M., Rakocevic, M., Caverzan, A., & Chavarria, G. (2017). Grain yield differences of soybean cultivars due to solar radiation interception. *American Journal of Plant Sciences*, 8(11), 2795-2810. <https://doi.org/10.4236/ajps.2017.811189>
- Mustofa, A., Zubaidah, S., & Kuswantoro, H. (2021). Correlation and path analysis on yield and yield components in segregating populations. *AIP Conference Proceedings*, 2353(1), Article 030030. <https://doi.org/10.1063/5.0052842>
- Paramesh, M., Reddy, D. M., Priya, M. S., Sumathi, P., Sudhakar, P., & Reddy, K. H. P. (2016). GT biplot analysis for yield and drought related traits in mung bean (*Vigna radiata* L. Wilczek). *Electronic Journal of Plant Breeding*, 7, 538-543. <https://doi.org/10.5958/0975-928X.2016.00069.7>
- Prvulović, D., Malenčić, D., & Miladinović, J. (2016). Antioxidant activity and phenolic content of soybean seeds extracts. *Agro-knowledge Journal*, 17(2), 121-132. <https://doi.org/10.7251/AGREN1602121P>
- Ramlal, A., Nautiyal, A., Lal, S. K., & Chigeza, G. (2023). A wonder legume, soybean: Prospects for improvement. *Frontiers in Plant Science*, 14, Article 1294185. <https://doi.org/10.3389/fpls.2023.1294185>
- Rivero-Cruz, J. F., Granados-Pineda, J., Pedraza-Chaverri, J., Pérez-Rojas, J. M., Kumar-Passari, A., Diaz-Ruiz, G., & Rivero-Cruz, B. E. (2020). Phytochemical constituents, antioxidant, cytotoxic, and antimicrobial activities of the ethanolic extract of Mexican brown propolis. *Antioxidants*, 9(1), Article 70. <https://doi.org/10.3390/antiox9010070>
- RStudio Team. (2020). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. <https://www.R-project.org/>

- Saeed, Z., Iqbal, S., Younas, U., Pervaiz, M., Bukhari, S. M., & Zaidi, A. (2020). Variation in antioxidant potential of *Vigna unguiculata* grown in pure and amended soil. *Kuwait Journal of Science*, 47(3), 2-14.
- Saito, H., Toyoda, Y., Hirata, H., Ota-Kontani, A., Tsuchiya, Y., Takada, T., & Suzuki, H. (2020). Soy isoflavone genistein inhibits an axillary osmidrosis risk factor ABCC11: In vitro screening and fractional approach for ABCC11-inhibitory activities in plant extracts and dietary flavonoids. *Nutrients*, 12(8), Article 2452. <https://doi.org/10.3390/nu12082452>
- Singh, S., Bhatt, P., Kumar, V., & Singh, N. P. (2024). Phytonutrients, anthocyanidins, and anthocyanins: Dietary and medicinal pigments with possible health benefits. In S. Nisha, D. Saini, R. J. Kesharwani, P. C. Gupta, & R. K. Keservani (Eds.), *Advances in flavonoids for human health and prevention of diseases* (pp. 23-46). Apple Academic Press. <https://doi.org/10.1201/9781003369813-2>
- Soedarjo, M. (2019). In vitro investigation on phenolic compound and antioxidant activities from methanolic extracts of black soybean seed. *International Journal of Research Studies in Agricultural Sciences*, 5(12), 1-7. <https://doi.org/10.20431/2454-6224.0512001>
- Sumardi, D., Pancoro, A., Yulia, E., Musfiroh, I., Prasetyono, J., Karuniawan, A., & Syamsudin, T. S. (2017). Potential of local black soybean as a source of the isoflavones daidzein and genistein. *International Food Research Journal*, 24(5), 2140-2145.
- Takahata, Y., Ohnishi-Kameyama, M., Furuta, S., Takahashi, M., & Suda, I. (2001). Highly polymerised procyanidins in brown soybean seed coat with a high radical-scavenging activity. *Journal of Agricultural and Food Chemistry*, 49(12), 5843-5847. <https://doi.org/10.1021/jf010307x>
- Tayade, R., Imran, M., Ghimire, A., Khan, W., Nabi, R. B. S., & Kim, Y. (2023). Molecular, genetic, and genomic basis of seed size and yield characteristics in soybean. *Frontiers in Plant Science*, 14, Article 1195210. <https://doi.org/10.3389/fpls.2023.1195210>
- Vu, T. T. H., Le, T. T. C., Vu, D. H., Nguyen, T. T., & Ngoc, T. (2019). Correlations and path coefficients for yield related traits in soybean progenies. *Asian Journal of Crop Science*, 11(1), 32-39. <https://doi.org/10.3923/ajcs.2019.32.39>
- Vyn, T. J., Yin, X., Bruusema, T. W., Jackson, C. J. C., Rajcan, I., & Brouder, S. M. (2002). Potassium fertilisation effects on isoflavone concentrations in soybean [*Glycine max* (L.) Merr.]. *Journal of Agricultural and Food Chemistry*, 50(12), 3501-3506. <https://doi.org/10.1021/jf0200671>
- Wang, X., Sun, J., Yi, Z., & Dong, S. (2025). Effects of seed size on soybean performance: Germination, growth, stress resistance, photosynthesis, and yield. *BMC Plant Biology*, 25(1), Article 219. <https://doi.org/10.1186/s12870-025-06224-3>
- Wu, H. J., Deng, J. C., Yang, C. Q., Zhang, J., Zhang, Q., Wang, X. C., Yang, F., Yang, W. Y., & Liu, J. (2017). Metabolite profiling of isoflavones and anthocyanins in black soybean [*Glycine max* (L.) Merr.] seeds by HPLC-MS and geographical differentiation analysis in Southwest China. *Analytical Methods*, 9(5), 792-802. <https://doi.org/10.1039/C6AY02970A>
- Yan, W., & Rajcan, I. R. (2002). Biplot analysis of test sites and trait relations of soybean in Ontario. *Canadian Journal of Plant Science*, 82(1), 11-20. <https://doi.org/10.2135/cropsci2002.1100>

- Yu, Y., Xing, Y., Zhang, Q., Zhang, Q., Huang, S., Li, X., & Gao, C. (2020). Soy isoflavone genistein inhibits hsa_circ_0031250/miR-873-5p/FOXO1 axis to suppress non-small-cell lung cancer progression. *IUBMB Life*, 73(1), 92-107. <https://doi.org/10.1002/iub.2404>
- Yusnawan, E. (2016). The diversity of secondary metabolites in Indonesian soybean genotypes. *Biodiversitas*, 17(2), 704-710. <https://doi.org/10.13057/biodiv/d170245>
- Zhang, J., Ge, Y., Han, F., Li, B., Yan, S., Sun, J., & Wang, L. (2014). Isoflavone content of soybean cultivars from maturity group 0 to VI grown in Northern and Southern China. *Journal of the American Oil Chemists' Society*, 91(6), 1019-1028. <https://doi.org/10.1007/s11746-014-2440-3>
- Zhang, R. F., Zhang, F. X., Zhang, M. W., Wei, Z. C., Yang, C. Y., Zhang, Y., Tang, X. J., Deng, Y. Y., & Chi, J. W. (2011). Phenolic composition and antioxidant activity in seed coats of 60 Chinese black soybean (*Glycine max* L. Merr.) varieties. *Journal of Agricultural and Food Chemistry*, 59(11), 5935-5944. <https://doi.org/10.1021/jf201593n>

Influence of Mother Palm Age and Harvesting Maturity Seed Nut on Seed Quality and Germination of Aromatic Pandan Coconut

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ABSTRACT

Aromatic Green Dwarf (AROD), commonly known in Malaysia as aromatic pandan coconut, is a dwarf type of coconut that has a unique aroma and flavour, and originated from Thailand. Aromatic pandan coconut is a popular coconut variety in Southeast Asia, particularly in Thailand and Malaysia, where it is widely consumed fresh. This popularity has led to an increasing demand for high-quality planting materials to support its commercial cultivation. Currently, the germination rate of aromatic pandan coconut seed nuts is around 40% under commercial practice, where matured seed nuts

were harvested at 12 months after pollination (MAP) for germination. Thus, this study was conducted to improve the seed nuts' germination performance by evaluating the effect of mother palm age and optimising the harvesting maturity stage of seed nuts harvested. In this study, aromatic pandan coconut seed nuts harvested at 10, 11, and 12 MAP were evaluated using two mother palm ages (10 years and 30 years). Seed germination performance was assessed at Tun Abdul Razak Agricultural Research Centre (Pusat Penyelidikan Pertanian Tun Abdul Razak, PPPTAR), Jengka, Pahang, using several parameters, including final germination rate after

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a four-month germination period, germination index, mean germination time, germination rate index, time spread of germination and seedling vigour index. The results showed that aromatic pandan coconut seed nuts harvested at 10 MAP achieved the highest final germination rate at 10 and 30 year-old mother palms, (60.7% and 65.3% respectively), followed by 11 MAP (56.0% and 51.3%) and 12 MAP (45.3% and 41.0%).

Keywords: Aromatic pandan coconut, harvesting maturity seed nut, seed germination

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a perennial tropical crop of major cultural, nutritional, and economic importance. It is widely known as the “tree of life” because almost every part of the palm can be utilised, including the stem for timber, leaves for roofing and handicrafts, sap for sugar and beverages, kernel for oil and milk, husk for fibre, and shell for charcoal and crafts (Mohd Effendi et al., 2023). Coconut palms are broadly classified into tall and dwarf types, in which tall varieties are mainly cross-pollinated, while dwarf varieties are largely self-pollinated (Perera et al., 2003). Among the dwarf coconuts, aromatic pandan coconut, known internationally as Aromatic Green Dwarf (AROD), is valued for its unique pandan-like aroma and flavour. Originating from Thailand, it is widely consumed fresh and considered a premium coconut in Southeast Asia and other coconut-growing regions (Bourdeix et al., 2010; Labouisse et al., 2005). The commercial planting of aromatic pandan coconut has grown quickly, increasing the need for high-quality planting materials, but the information on the germination behaviour and seed quality is still limited. In general, basic knowledge on palm seed dormancy and germination, including in coconut, also remains limited (Baskin & Baskin, 2014). Although recent advances in embryo culture and somatic embryogenesis demonstrate that clonal propagation of coconut is technically possible, these methods remain limited in large-scale field application. Therefore, commercial coconut production continues to depend mainly on seed nuts, making seed quality and germination performance critical for nursery success and field establishment. Seed germination is an important stage in coconut propagation because coconut seeds are recalcitrant and continue to remain physiologically active after harvest. It is also affected by maternal and developmental factors (Baskin & Baskin, 2014; Beveridge et al., 2022; Paull & Ketsa, 2015). Coconut produces a large, effectively viviparous seed, in which the embryo continues to develop after fruit maturity and rapidly loses viability when moisture is reduced (Harries, 2012). Fruit development generally requires about 330 to 420 days after pollination, after which the embryo nuts harvested too early may not be physiologically mature, while over-mature nuts may lose moisture and become less viable due to metabolic changes. (Beveridge et al., 2022). Evidence from other palms supports the importance of the harvest stage and internal seed condition. In oil palm (*Elaeis guineensis*), seeds harvested at optimal maturity

stages show higher germination percentages and more uniform emergence than those collected too early or too late (Norsazwan et al., 2023). Similar patterns have been reported in macaw palm (*Acrocomia aculeata*), in which delayed or irregular germination is closely linked to physiological dormancy and hormonal imbalance within the seed (Ribeiro et al., 2015) and in date palm (*Phoenix dactylifera*), where seed maturity and moisture status also determine germination success and early seedling growth (Mohammed & Said, 2018). These findings indicate that, within the Arecaceae, harvest stage and internal seed condition are key determinants of seed performance. In addition to harvest maturity, characteristics associated with the mother palm may influence seed quality. In perennial crops and palms, seed size, nutrient reserve, and early seedling vigour often vary with plantation age. Several studies report superior seed characteristics from palms in their optimum productive stage compared with very young or old stands (Kome & Tabi, 2020; Lyngdoh et al., 2014). Similar patterns have been reported in other long-lived species, where middle-aged mother plants tend to produce offspring with higher vigour and better early growth, and very old plants may show declining reproductive performance (Alejano et al., 2019; Pardos et al., 2022). However, there is still a lack of information on how mother palm age affects coconut seed germination, particularly for high-value varieties such as aromatic pandan coconut. Most nursery practices rely on general recommendations without considering physiological differences among palms of different ages. The present study addresses this gap by evaluating the combined effects of mother palm age and nut harvest maturity on seed performance in aromatic pandan coconut. Two palm ages were selected to represent different physiological stages of production, which are 10-year-old palms corresponding to the early productive phase and 30-year-old palms representing fully mature palms in commercial plantations. Nuts were harvested at 10, 11, and 12 months after pollination based on the normal development period of coconut fruits. In commercial practice, seed nuts are usually harvested at around 12 months after pollination, while 10 and 11 months represent stages just before full maturity. These stages were included to evaluate whether earlier harvesting within the mature fruit window could improve germination performance (Beveridge et al., 2022; Paull & Ketsa, 2015). The findings are expected to identify suitable harvesting stages and palm age sources for producing high-quality planting materials, thereby providing practical guidance for nursery management and sustainable cultivation of these high-value coconut varieties.

MATERIALS AND METHODS

Plant Material and Experimental Site

The study was conducted at the Aromatic Pandan Coconut Seed Garden and Nursery operated by FGV Agri Services at the Tun Abdul Razak Agricultural Research Centre (Pusat Penyelidikan Pertanian Tun Abdul Razak, PPPTAR), Jengka, Pahang, Malaysia (3.8822° N, 102.5315° E).

The seed garden consisted of around 2,400 aromatic pandan coconut palms managed under standard commercial practices. Seed germination and nursery management were carried out at the Aromatic Pandan Coconut Nursery within the same research complex. Aromatic pandan coconut is a self-pollinated dwarf variety, and controlled hand pollination was not performed. All seed nuts resulted from natural pollination under field conditions.

Seed Nut Sampling and Harvest Maturity Evaluation

Aromatic pandan coconut seed nuts were collected from the study site described above. Seed nuts were harvested from mother palms of two age groups (10 and 30 years after planting) at three maturity stages, namely 10, 11, and 12 months after pollination (MAP). Harvest maturity was determined based on exocarp colour following the Department of Agriculture (DOA) Malaysia Standard Operating Procedure for the Production of Aromatic Pandan Coconut Seedlings (DOA, 2011). To validate the relationship between nut age and exocarp colour, 100 aromatic pandan coconut inflorescences (bunches) were tagged at pollination and monitored daily to observe changes in exocarp colour from the button stage through to the development of a dark brown exocarp, corresponding to nut maturity. Seed nuts were harvested randomly from multiple bunches of aromatic pandan coconut across the seed garden to minimise sampling bias. Figure 1 shows the images of seed nuts at three maturity stages.

Nursery Preparation and Seed Sowing

Seed nuts were sown in a shaded nursery under 50% shade netting following the Standard Operating Procedure for Aromatic Pandan Coconut Seedling Production (DOA, 2011). Prior to sowing, seed nuts were mechanically scarified to facilitate water uptake and promote uniform germination. Scarification was performed by carefully removing excess husk material around the germination pore region without damaging the endocarp or embryo.



Figure 1. Aromatic pandan coconut seed nuts harvested at 10, 11, and 12 months after pollination (left to right), showing differences in exocarp colour with increasing maturity stage

This procedure was applied uniformly to all seed nuts in line with the Department of Agriculture (DOA) Malaysia guidelines, after which the seed nuts were immediately sown on sand beds. Nursery beds were prepared using raised sand beds to ensure sufficient drainage and uniform moisture conditions, with seed nuts placed horizontally in the sand. Irrigation was supplied using a mist irrigation system applied twice daily in the morning (0800-0900 h) and evening (1600-1700 h). Each irrigation cycle lasted approximately 10-15 minutes, maintaining consistently moist but non-waterlogged conditions throughout the germination period. No fertiliser was applied during germination, and weed control was carried out manually as required.

Experimental Design

Three levels of seed nut maturity (10, 11, and 12 months after pollination) and two mother palm age groups (10 and 30 years) were evaluated using a factorial randomised complete block design (RCBD). The experiment consisted of six treatment combinations with three replications of 100 seed nuts for each treatment, giving a total of 1,800 seed nuts used in the study. The sand beds were arranged into blocks according to the direction of sunlight exposure within the nursery to minimise environmental variation.

Seed Germination Assessment

Germination was monitored weekly for 16 weeks after sowing. A seed nut was considered germinated upon visible emergence of the plumule. Final germination percentage (FGP) was determined 16 weeks after sowing, when germination had stabilised, and no further seed emergence was observed. This was consistent with standard nursery practices for aromatic pandan coconut and reported coconut germination behaviour (Beveridge et al., 2022; DOA, 2011). Germination parameters were calculated following the method described by Ranal et al. (2009). These parameters included final germination percentage (FGP), germination index (GI), mean germination time (MGT), germination rate index (GRI), time spread of germination (TSG), and seedling vigour index (SVI), as defined in the equations in Table 1.

Seed Morphological Measurements

Morphological analysis was conducted using nine seed nuts randomly selected for each mother palm age across the three maturity stages (three nuts per maturity stage), resulting in 18 seed nuts for morphological analysis. Data were collected on fruit dimensions, fruit mass, volume, component mass and component thickness of aromatic pandan coconut, as illustrated in Figure 2. Husk thickness was measured by cutting each fruit longitudinally and recording measurements at four fixed positions around the fruit (H1-H4: top, bottom, and two side positions).

Table 1
List of equations and parameters

No.	Parameter	Formula and description	
1	Final germination percentage, FGP (%)	$FGP = \frac{\text{Total seeds germinated at the end of the trial}}{\text{Number of initial seeds used}} \times 100\%$	[1]
2	Germination index, GI (%/day)	$GI = \sum_{i=1}^n \frac{G_i}{D_i}$ where G_i is the number of seeds germinated on day D_i and D_i is the i^{th} day of germination	[2]
3	Mean germination time, MGT (day)	$MGT = \frac{\sum Fx}{\sum F}$ where F is the number of seeds germinated on day x	[3]
4	Germination rate index, GRI (% / day)	$GRI = \frac{G_1}{1} + \frac{G_2}{2} + \dots + \frac{G_i}{i}$ where G_i is the germination percentage on i^{th} day.	[4]
5	Time spread of germination, TSG (day)	$TSG = \text{Final day of germination} - \text{First day of germination}$	[5]
6	Seedling vigour index, SVI	$SVI = \text{Seedling length (cm)} \times \text{germination (\%)} $	[6]

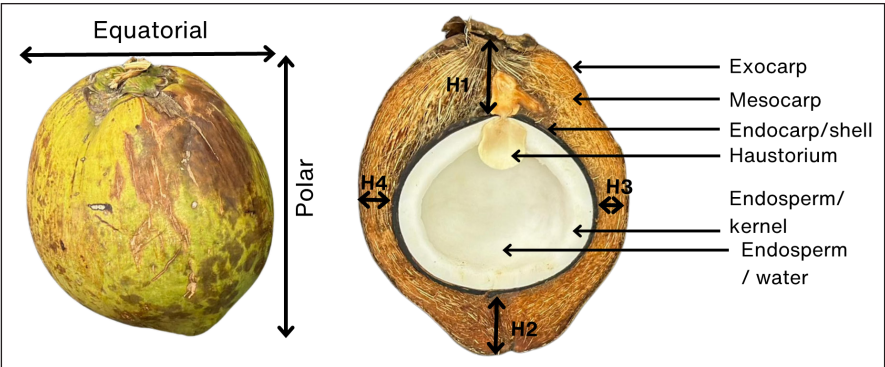


Figure 2. Fruit dimensional and component thickness parameters of aromatic pandan coconut

The average of H1-H4 was used as the husk thickness for each fruit. Kernel thickness was measured at one standard position next to the endosperm, as shown in Figure 2, because the kernel layer is generally uniform around the fruit.

Seedling Physiological Measurements

Seedling physiological parameters were measured at 16 weeks after sowing using a LI-COR LI-6400XT Portable Photosynthesis System (LI-COR, Inc., Lincoln, Nebraska, USA). The parameters measured included net photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), intercellular CO_2 concentration ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) and transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$). For each treatment, three healthy and uniformly growing seedlings were randomly selected from the germinated population. Measurements were taken from the middle portion of the third fully expanded leaf, with five readings recorded per leaf to obtain an average value per seedling. Data collection was conducted between 0800 and 1100h to minimise potential variation associated with midday stomatal closure.

Statistical Analysis

Data were analysed using two-way analysis of variance (ANOVA) with a factorial Randomised Complete Block Design (RCBD) to evaluate the main effects of mother palm age (10 and 30 years) and harvesting maturity stage (10, 11 and 12 months after pollination) as well as their interaction on seed germination, seed morphological traits and seedling physiological parameters. Normality and homogeneity of variance were verified using Shapiro-Wilk and Levene's tests, respectively. When significant effects were detected, treatment means were compared using Tukey's Honest Significant Difference (HSD) test at a 5% significance level ($P \leq 0.05$). All statistical analyses were performed using R software and XLSTAT.

RESULTS

Seed Germination

In the present study, findings showed that harvesting maturity strongly affected final germination percentage (FGP) and mean germination time (MGT), while palm age had a smaller effect on seed germination parameters. The interaction between palm age and harvesting maturity was mainly seen for FGP and MGT ($p < 0.05$), indicating that the effect of harvesting stage showed small differences with the age of the mother palm. Seeds harvested at 10 months produced the highest germination for both palm ages. The highest FGP was recorded from 30-year-old palms at 10 months (65.33%), followed by 10-year-old palms at the same stage (60.67%) (Table 2).

Table 2
Final germination percentage (FGP), germination index (GI), germination index rate (GRI), mean germination time (MGT), time spread of germination (TSG) and seedling vigour index (SVI) for seeds germinated at 10, 11 and 12 months after pollination at 10 years and 30 years of mother palm age value were presented in mean ±standard error (SE)

Palm Age (years)	Harvesting Maturity (months)	FGP (%)	GI	GRI (%/day)
10	10	60.67 ± 2.73 ^{ab}	1.02 ± 0.07 ^a	6.04 ± 0.50 ^a
	11	56.00 ± 4.58 ^{abc}	1.47 ± 0.02 ^a	8.97 ± 0.21 ^a
	12	45.33 ± 0.88 ^{bc}	1.84 ± 0.22 ^a	9.63 ± 0.76 ^a
30	10	65.33 ± 2.91 ^a	1.15 ± 0.07 ^a	7.03 ± 0.43 ^a
	11	51.33 ± 1.76 ^{abc}	1.46 ± 0.04 ^a	8.57 ± 0.21 ^a
	12	41.00 ± 7.21 ^c	1.95 ± 0.41 ^a	9.69 ± 2.03 ^a

Palm Age (years)	Harvesting Maturity (months)	MGT (day)	TSG (day)	SVI
10	10	63.00 ± 2.08 ^a	65.33 ± 6.17 ^a	2908.90 ± 201.60 ^a
	11	44.33 ± 1.86 ^b	72.33 ± 6.17 ^a	3256.11 ± 194.18 ^a
	12	35.67 ± 2.73 ^{bc}	77.00 ± 10.69 ^a	2869.14 ± 134.16 ^a
30	10	59.33 ± 0.67 ^a	67.67 ± 6.17 ^a	3440.85± 183.14 ^a
	11	43.33± 2.33 ^b	84.00± 4.04 ^a	3092.10± 89.78 ^a
	12	32.33 ± 2.73 ^c	86.33 ± 12.35 ^a	2728.08 ± 625.51 ^a

Note. Means followed by different letters within the same column across all treatments (all palm ages and harvesting maturities) are significantly different at $p < 0.05$ (Tukey's HSD test)

In contrast, seeds harvested at 12 months showed the lowest FGP for both 10-year-old (45.33%) and 30-year-old palms (41.00%). This pattern clearly shows that later harvesting reduces germination regardless of palm age. Germination index (GI) and germination rate index (GRI) did not differ significantly among treatments. Although these values increased slightly with harvesting maturity, the differences were not significant, indicating that the speed of germination was similar across all treatments. Mean germination time (MGT) was strongly influenced by harvesting maturity. Seeds harvested at 10 months took the longest time to germinate, with 63.00 ± 2.08 days for 10-year-old palms and 59.33 ± 0.67 days for 30-year-old palms. In contrast, seeds harvested at 12 months germinated much faster with MGT values of 35.67 ± 2.73 days and 32.33 ± 2.73 days for 10 and 30-year-old palms, respectively. This shows that more mature seeds begin germination earlier, even though fewer seeds finally germinate.

Time spread of germination (TSG) and seedling vigour index (SVI) showed no significant differences among treatments. No interaction between palm age and harvesting maturity was detected for these parameters.

Seed Morphology

Fruit Dimensional Parameters

Table 3 summarises the dimensional parameters of aromatic pandan coconut in relation to mother palm age and harvesting maturity stage. Statistical analysis showed that mother palm age had a significant effect ($p < 0.05$) on all measured dimensional parameters, including equatorial circumference, polar circumference, equatorial diameter, and polar diameter. In contrast, harvesting maturity stage (10, 11, and 12 months after pollination) did not significantly affect these parameters ($p > 0.05$), and no significant interaction between palm age and harvesting maturity was detected ($p > 0.05$). Across all maturity stages, fruits from 30-year-old palms were consistently larger than those from 10-year-old palms. For the 30-year-old palms, fruit dimensions generally increased with advancing harvesting maturity, with the highest values for equatorial circumference (49.30 ± 0.64 cm), polar circumference (51.71 ± 0.94 cm), and polar diameter (16.77 ± 0.44 cm) recorded at 12 months after pollination. In contrast, fruits from 10-year-old palms showed smaller dimensions and no clear increasing trend with harvesting maturity, indicating more limited fruit expansion in younger palms.

Table 3
Dimensional parameters of aromatic pandan coconut fruit harvested at 10, 11 and 12 months after pollination from 10 and 30-year-old mother palms (mean $\pm$ SE)

Palm Age (years)	Harvesting Maturity (months)	Equatorial Circumference (cm)	Polar Circumference (cm)	Equatorial Diameter (cm)	Polar Diameter (cm)
10	10	47.53 $\pm$ 0.55 ^{ab}	49.02 $\pm$ 0.50 ^{ab}	14.77 $\pm$ 0.16 ^{ab}	15.61 $\pm$ 0.30 ^b
	11	45.53 $\pm$ 1.29 ^b	47.84 $\pm$ 1.07 ^b	13.83 $\pm$ 0.41 ^b	15.58 $\pm$ 0.28 ^b
	12	44.81 $\pm$ 0.62 ^b	47.64 $\pm$ 0.59 ^b	13.82 $\pm$ 0.33 ^b	15.53 $\pm$ 0.33 ^b
30	10	48.66 $\pm$ 0.42 ^a	50.38 $\pm$ 0.39 ^a	15.08 $\pm$ 0.17 ^a	16.68 $\pm$ 0.23 ^a
	11	49.11 $\pm$ 0.63 ^a	50.50 $\pm$ 0.87 ^a	15.07 $\pm$ 0.22 ^a	16.30 $\pm$ 0.35 ^a
	12	49.30 $\pm$ 0.64 ^a	51.71 $\pm$ 0.94 ^a	14.88 $\pm$ 0.19 ^{ab}	16.77 $\pm$ 0.44 ^a

Note. Means followed by different letters within the same column across all treatments (all palm ages and harvesting maturities) are significantly different at $p < 0.05$ (Tukey’s HSD test)

Fruit mass, Volume, and Component Mass

The fruit mass, volume and component mass are summarised in Table 4. The results showed that mother palm age and harvesting maturity significantly affected most parameters ($p < 0.05$),

with a significant interaction for selected components. Total fruit mass decreased with increasing harvesting maturity in both palm ages. In 10-year palms, fruit mass declined from 874.89 g at 10 MAP to 579.89 g at 12 MAP, while in 30-year palms it decreased from 868.11 g to 726.89 g. This reduction was mainly due to a strong decrease in husk mass across maturity stages. Harvesting maturity also significantly affected dehusked fruit mass without water, but the pattern differed by palm age. In 10-year palms, values decreased slightly with maturity, whereas in 30-year palms they increased at 11 to 12 MAP compared with 10 MAP. This shows that later maturity reduces husk contribution, but solid internal mass is better maintained in fruits from older palms.

Table 4
Palm age differences in fruit mass and volume of the component (husk, kernel, and shell) mass of aromatic pandan coconut fruit value were presented in mean $\pm$ standard error

Palm Age (years)	Harvesting Maturity (months)	Fruit Mass (g)	Fruit Mass without Water (g)
10	10	874.89 $\pm$ 17.74 ^a	735.56 $\pm$ 16.48 ^a
	11	698.22 $\pm$ 81.75 ^{ab}	554.89 $\pm$ 32.20 ^{bc}
	12	579.89 $\pm$ 24.78 ^b	540.22 $\pm$ 19.86 ^c
30	10	868.11 $\pm$ 13.93 ^a	745.22 $\pm$ 11.41 ^a
	11	854.67 $\pm$ 49.08 ^a	695.89 $\pm$ 36.92 ^a
	12	726.89 $\pm$ 26.74 ^{ab}	648.22 $\pm$ 19.70 ^{ab}

Palm Age (years)	Harvesting Maturity (months)	Dehusked Fruit without Water (g)	Mass of Fruit Husk (g)
10	10	384.22 $\pm$ 8.84 ^{bc}	351.33 $\pm$ 15.79 ^a
	11	355.33 $\pm$ 19.53 ^{bc}	199.56 $\pm$ 19.76 ^c
	12	353.89 $\pm$ 12.52 ^c	186.33 $\pm$ 7.63 ^c
30	10	400.67 $\pm$ 9.18 ^{ab}	344.56 $\pm$ 7.38 ^a
	11	431.00 $\pm$ 14.53 ^a	264.89 $\pm$ 23.07 ^b
	12	410.67 $\pm$ 13.37 ^a	237.56 $\pm$ 9.50 ^b

Note. Means followed by different letters within the same column across all treatments (all palm ages and harvesting maturities) are significantly different at $p < 0.05$ (Tukey’s HSD test)

Husk and Kernel Thickness

The ANOVA findings and summary of coconut component thickness are stated in Table 5. The result showed that shell, kernel, H2 and H4 thickness are not significantly affected by

palm age and harvesting maturity stage ($p>0.05$), while the H1 and H3 thickness have a significant effect ($p<0.05$) in palm age. As the stage of maturity in harvesting increased, shell and kernel thickness for 30-year-old palm age of coconut also increased, while the 10-year-old palm age coconut showed decreased thickness in shell and kernel dimensions. The 30-year-old palm age coconut in 12 months of maturity stage at harvesting has the highest thickness in shell ($3.44\pm0.51\text{mm}$) and kernel ($11.66\pm1.29\text{mm}$). Mature coconut showed the highest husk thicknesses, H1 ($4.40\pm1.01\text{mm}$), H2 ($3.12\pm0.45\text{mm}$) in 12 months' maturity of harvesting, while H3 ($2.07\pm0.26\text{mm}$) and H4 ($25.25 \pm 4.23 \text{ mm}$) were highest in 11 months and 10 months' maturity of harvesting.

Table 5
Palm age differences in the thickness of the components (husk, kernel, and shell) of aromatic pandan coconut fruit value were presented in mean $\pm$ standard error

Palm Age (years)	Harvesting Maturity (months)	Husk Thickness, H1 (cm)	Husk Thickness, H2 (cm)	Husk Thickness, H3 (cm)	Husk Thickness, H4 (cm)
10	10	4.08 ± 0.24^{ab}	2.51 ± 0.11^b	1.97 ± 0.10^{ab}	1.93 ± 0.09^a
	11	3.92 ± 0.16^{ab}	2.63 ± 0.18^{ab}	1.66 ± 0.08^{ab}	1.82 ± 0.13^a
	12	3.47 ± 0.18^b	2.79 ± 0.09^{ab}	1.56 ± 0.09^b	1.79 ± 0.10^a
30	10	4.26 ± 0.18^a	2.58 ± 0.11^{ab}	1.91 ± 0.09^{ab}	2.02 ± 0.10^a
	11	4.04 ± 0.28^{ab}	2.72 ± 0.17^{ab}	2.07 ± 0.09^a	1.86 ± 0.12^a
	12	4.40 ± 0.34^a	3.12 ± 0.15^a	1.99 ± 0.15^a	1.97 ± 0.12^a

Palm Age (years)	Harvesting Maturity (months)	Shell Thickness (mm)	Kernel Thickness (mm)
10	10	2.82 ± 0.22^{ab}	11.24 ± 0.32^a
	11	2.80 ± 0.06^{ab}	11.02 ± 0.85^a
	12	2.58 ± 0.20^b	10.61 ± 0.29^a
30	10	2.61 ± 0.28^b	11.03 ± 0.12^a
	11	2.91 ± 0.12^{ab}	11.02 ± 0.41^a
	12	3.44 ± 0.17^a	11.66 ± 0.43^a

Note. Means followed by different letters within the same column across all treatments (all palm ages and harvesting maturities) are significantly different at $p < 0.05$ (Tukey's HSD test)

Seedling Physiology

The ANOVA showed that there is no significant relationship ($p>0.05$) of mother palm age with nut harvesting maturity stage on all physiology parameter, as shown in Table 6. The net photosynthesis values ranged from 8.21 to 11.28 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for 10-year-old palm age, and 11.73 to 13.18 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for seeds harvested from 30-year-old palm age. A similar pattern was observed for stomatal conductance, intercellular concentration and transpiration rate parameters.

Table 6
Net photosynthesis, stomatal conductance, intercellular concentration and transpiration rate for seeds germinated at 10, 11 and 12 months after pollination at 10-year-old and 30-year-old mother palm ages were presented in mean $\pm$ standard error

Palm Age (years)	Harvesting Maturity (months)	Net Photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Stomatal Conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
10	10	10.95 $\pm$ 0.11 ^a	1.02 $\pm$ 0.07 ^a
	11	8.21 $\pm$ 2.38 ^a	1.47 $\pm$ 0.02 ^a
	12	11.28 $\pm$ 1.92 ^a	1.84 $\pm$ 0.22 ^a
30	10	11.73 $\pm$ 0.42 ^a	1.15 $\pm$ 0.07 ^a
	11	13.02 $\pm$ 0.49 ^a	1.46 $\pm$ 0.04 ^a
	12	13.18 $\pm$ 0.53 ^a	1.95 $\pm$ 0.41 ^a

Palm Age (years)	Harvesting Maturity (months)	Intercellular concentration ($\mu\text{mol CO}_2 \text{ mol}^{-1}$)	Transpiration rate ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
10	10	257.73 $\pm$ 20.11 ^a	0.002 $\pm$ 3.280 $\times 10^{-4}$ ^a
	11	260.36 $\pm$ 9.43 ^a	0.001 $\pm$ 7.569 $\times 10^{-4}$ ^a
	12	203.96 $\pm$ 40.85 ^a	0.002 $\pm$ 7.064 $\times 10^{-4}$ ^a
30	10	298.94 $\pm$ 7.26 ^a	0.003 $\pm$ 3.461 $\times 10^{-4}$ ^a
	11	271.37 $\pm$ 7.96 ^a	0.003 $\pm$ 3.404 $\times 10^{-4}$ ^a
	12	273.26 $\pm$ 13.10 ^a	0.003 $\pm$ 3.578 $\times 10^{-3}$ ^a

Note. Means followed by different letters within the same column across all treatments (all palm ages and harvesting maturities) are significantly different at $p < 0.05$ (Tukey’s HSD test)

DISCUSSION

The present study demonstrates that harvesting maturity is the primary factor influencing seed germination in aromatic pandan coconut (*Cocos nucifera* L.), regardless of mother palm age. Nuts harvested at 10 months after pollination consistently produced the highest

final germination percentage (FGP) for both 10 and 30-year-old palms. This directly supports the conclusion that 10 months is the optimal harvesting stage for seed nuts, as it yields the highest percentage of viable seeds. Although seeds harvested at later stages showed shorter mean germination time (MGT), their FGP was significantly lower. Hence, faster emergence at later maturity does not reflect better seed quality. Based on the present data, 10 months provides the best balance between high germination success and acceptable germination behaviour.

Coconut is a recalcitrant seed, and its viability is closely linked to the physiological condition at harvest. Paull and Ketsa (2015) reported that coconut reaches physiological maturity between 10 and 12 months after pollination. Delaying harvesting for the palm beyond this optimal period may cause nuts to age, dehydrate, and undergo biochemical degradation, all of which reduce embryo viability. Ibrahim et al. (2022) similarly showed that coconut seeds harvested beyond the optimal stage recorded reduced germination due to ageing and moisture loss. These mechanisms are characteristic of recalcitrant seeds, which rapidly lose viability when physiological integrity declines. Comparable responses have been reported in other perennial palms. Norsazwan et al., (2023) demonstrated that oil palm seeds harvested at the optimal stage (20 weeks after pollination) achieved the highest germination (85.1%) compared to the 70.5% observed at 18 WAP and 80.9% at 22 WAP. This closely mirrors the present findings, where the highest FGP occurred at approximately 10 months. This evidence suggests that the optimal maturity stage for harvesting oil palm seeds aligns closely with the observation in the current study, where the highest final germination percentage (FGP) was noted for nuts collected at around 10 months. Therefore, both studies conclude that harvest maturity plays a critical role in seed viability, reinforcing the idea that optimal harvesting can yield seeds with greater germination performance.

Fruit morphology in coconut varies widely among cultivars and developmental stages (Kouadio et al., 2023; Rajesh et al., 2014). In the present study, fruit dimensional and mass-related parameters were strongly influenced by mother palm age. Fruits from 30-year-old palms were consistently larger and heavier across all maturity stages, indicating a greater capacity for assimilate supply and fruit filling in older palms. In contrast, fruits from 10-year-old palms showed reduced dimensions with advancing maturity, suggesting that younger palms may experience limitations in sustaining fruit growth during later developmental stages. Similar increases in equatorial and polar circumferences with advancing maturity across coconut varieties have been reported by Kouadio et al. (2023), supporting the patterns observed in this study. Across both palm ages, fruit mass and component mass declined with increasing harvesting maturity, particularly for husk and water content. Beveridge et al. (2022) reported that coconut water volume decreases as nuts mature, with internal spaces gradually replaced by gases, explaining the reduction in fruit mass observed at later stages. In this study, husk and kernel thickness were highest

at 12 months, confirming that later harvesting stages are associated with thicker structural components, as also reported by Kouadio et al. (2023). Harries (1981) and Odufale et al. (2024) showed that fruit weight, size, and coconut water volume are positively correlated with germination, whereas husk thickness, husk weight, and shell thickness are negatively correlated. A similar pattern was observed in this study, where nuts harvested at 10 months had greater fruit mass and produced higher germination than those harvested at 12 months. However, the relationship was not uniform across all fruit components, suggesting that harvest stage rather than size alone determines germination success.

Despite clear differences in fruit morphology and seed performance, seedling physiological traits, including net photosynthesis, stomatal conductance, intercellular CO₂ concentration, and transpiration, were not significantly affected by palm age or harvesting maturity, nor by the interaction between both factors. The values recorded fall within the range commonly reported for coconut seedlings, which is approximately 2-15 µmol CO₂ m⁻² s⁻¹ (Jayasekara et al., 2010). Stable photosynthetic performance across treatments indicates that, once germination has occurred and seedlings establish functional leaves, physiological activity becomes relatively uniform. This suggests that maternal and developmental effects are observed mainly at the fruit and seed level and do not translate into early physiological disadvantages during seedling establishment.

CONCLUSION

This study demonstrates that harvesting maturity is the primary factor influencing germination performance of aromatic pandan coconut seed nuts, while mother palm age has only a minor effect. Nuts harvested at 10 months after pollination consistently produced the highest final germination percentage for both 10 and 30-year-old palms. However, seeds harvested at later stages germinated in a shorter time but resulted in lower final germination percentages, indicating a relationship between germination speed and total germination success. It is therefore suggested to harvest the seed nut at 10 months after pollination to obtain a higher final germination percentage.

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REFERENCES

- Alejano, R., Dominguez-Delmás, M., García-González, I., Wazny, T., Vázquez-Piqué, J., & Fernández-Martínez, M. (2019). The age of black pine (*Pinus nigra* Arn. ssp. *salzmannii* (Dunal) Franco) mother trees has

- no effect on seed germination and on offspring seedling performance. *Annals of Forest Science*, 76(1), Article 15. <https://doi.org/10.1007/s13595-019-0801-7>
- Baskin, J. M., & Baskin, C. C. (2014). What kind of seed dormancy might palms have? *Seed Science Research*, 24(1), 17-22. <https://doi.org/10.1017/S0960258513000342>
- Beveridge, F. C., Kalaipandian, S., Yang, C., & Adkins, S. W. (2022). Fruit biology of coconut (*Cocos nucifera* L.). *Plants*, 11(23), Article 3293. <https://doi.org/10.3390/plants11233293>
- Bourdeix, R., Batugal, P., Oliver, J. T., & George, M. L. (Eds.). (2010). *Catalogue of conserved coconut germplasm*. International Coconut Genetic Resources Network (COGENT), Bioversity International, Regional Office for Asia, the Pacific and Oceania. <https://cgspace.cgiar.org/items/71965791-3fa8-495a-a479-60135f72c853>
- Department of Agriculture Malaysia [DOA]. (2011). *Standard Operating Procedure (SOP) Pengeluaran Anak Benih Kelapa Pandan* Standard Operating Procedure (SOP) pengeluaran anak benih kelapa pandan [Standard operating procedure (SOP) for the production of Pandan coconut seedlings].
- Harries, H. C. (1981). Germination and taxonomy of the coconut. *Annals of Botany*, 48(6), 873-883. <https://doi.org/10.1093/oxfordjournals.aob.a086195>
- Harries, H. C. (2012). Germination rate is the significant characteristic determining coconut palm diversity. *AoB Plants*, Article pls045. <https://doi.org/10.1093/aobpla/pls045>
- Ibrahim, K. M., Olanrewaju, O. O., Chuks, A. V., Abdulrahman, Y. S., & Mubo, A.-A. A. (2022). Effect of sorting and spacing on the germination of coconut ground bed nursery. *Innovare Journal of Agricultural Sciences*, 10(5), 1-3. <https://doi.org/10.22159/ijags.2022.v10i5.46153>
- Jayasekara, C., Nainanayake, N. P. A. D., & Jayasekara, K. S. (2010). Photosynthetic characteristics and productivity of the coconut palm. *Cocos*, 11, 07-20. <https://doi.org/10.4038/cocos.v11i0.2149>
- Kome, G. K., & Tabi, F. O. (2020). Towards sustainable oil palm plantation management: Effects of plantation age and soil parent material. *Agricultural Sciences*, 11(01), 54-70. <https://doi.org/10.4236/as.2020.111004>
- Kouadio, M. K., Konan, K. J. L., Doubi, B. T. S., Soro, P. L., Djaha, K. E., & Koné, F. M. T. (2023). Fruit morphological characteristics at different maturity stages of coconut (*Cocos nucifera* L.) improved hybrids (PB113+, PB121+) and their parent males (RIT+, WAT+). *African Journal of Agricultural Research*, 19(10), 987-993. <https://doi.org/10.5897/AJAR2023.16461>
- Labouisse, J. P., Sileye, T., Morin, J. P., Hamelin, C., Baudouin, L., Bourdeix, R., & Rouzière, A. (2005). Coconut (*Cocos nucifera* L.) genetic improvement in Vanuatu: Overview of research achievements from 1962 to 2002. Part 2: Improvement of the Vanuatu Tall by hybridisation. *OCL - Oleagineux Corps Gras Lipides*, 12(2), 170-179. <https://doi.org/10.1051/ocl.2005.0170>
- Lyngdoh, N., Kumar, M., Kumar, N., & Pandey, A. K. (2014). Effect of age of plantation on seed characters and growth performance of Tokopatta (*Livistona jenkinsiana* Griff.) seedling. *Journal of Applied and Natural Science*, 6(2), 672-676. <https://doi.org/10.31018/jans.v6i2.515>
- Mohammed, N. M. I., & Said, A. G. E. (2018). Date palm (*Phoenix dactylifera*) seeds germination. *Cell Biology and Development*, 2(2), 63-68. <https://doi.org/10.13057/cellbioldev/v020203>

- Mohd Effendi, M. N., Norma Hussin, Mirfat Ahmad Hasan Salahuddin, Asraf Hadi Abu Samah, Muhammad Faidhi Towhid, & Muhammad Faris Mohd Radzi. (2023). Evaluation of phenolic content and antibacterial activity of coconut (*Cocos nucifera* L.) shell and coir powder in different extraction solvents. *Journal of Tropical Plant Physiology*, 15(1), Article 9. <https://doi.org/10.56999/jtpp.2023.15.1.28>
- Mohd Norsazwan, G., Uma Rani, S., & Parameswari, N. (2023). Oil palm (*Elaeis Guineensis*) seed characteristics and germination potential as influenced by maturity stage and fruitlet position on a bunch. *Journal of Oil Palm Research*, 36(1), 94-103. <https://doi.org/10.21894/jopr.2023.0014>
- Odufale, O. O., Koloche, M. I., Ahanon, M. J., & Nwite, A. P. (2024). Evaluating the variability of germination pattern of coconut types. *Greener Journal of Agricultural Sciences*, 14(2), 123-127. <https://doi.org/10.15580/GJAS.2024.2.051224060>
- Pardos, M., Vázquez-Piqué, J., Benito, L., Madrigal, G., Alejano, R., Fernández, M., & Calama, R. (2022). Does the age of *Pinus sylvestris* mother trees influence reproductive capacity and offspring seedling survival? *Forests*, 13(6), 1-13. <https://doi.org/10.3390/f13060937>
- Paull, R. E., & Ketsa, S. (2015). Coconut: Postharvest quality-maintenance guidelines. *Fruit, Nut and Beverage Crops*, 1-5. <https://www.ctahr.hawaii.edu/site/PubList.aspx?key=Fruit,%20Nut,%20and%20Beverage%20Crops>
- Perera, L., Russell, J. R., Provan, J., & Powell, W. (2003). Studying genetic relationships among coconut varieties/populations using microsatellite markers. *Euphytica*, 132(1), 121-128. <https://doi.org/10.1023/A:1024696303261>
- Rajesh, M. K., Samsudeen, K., Rejusha, P., Manjula, C., Rahman, S., & Karun, A. (2014). Characterisation of Annur and Bedakam ecotypes of coconut from Kerala state, India, using microsatellite markers. *International Journal of Biodiversity*, 2014(1), 1-7, Article 260895. <https://doi.org/10.1155/2014/260895>
- Ribeiro, L. M., Garcia, Q. S., Müller, M., & Munné-Bosch, S. (2015). Tissue-specific hormonal profiling during dormancy release in macaw palm seeds. *Physiologia Plantarum*, 153(4), 627-642. <https://doi.org/10.1111/ppl.12269>

Performance of Maltodextrin-pectin-based Edible Coating with Lemongrass Essential Oil for Extending the Shelf Life of Fresh Tomatoes

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ABSTRACT

Tomatoes are highly perishable fruits that continue metabolic and respiratory activities after harvest, leading to nutrient degradation and rapid spoilage. Edible coatings offer an environmentally friendly solution owing to their biodegradability and low environmental impact. This study examined the effect of incorporating lemongrass essential oil (EO) into a maltodextrin-pectin-based protective coating on tomato quality and shelf life. The protective coating was applied by spraying after 20 days at temperatures between 30 °C and 4 °C. Both coated and uncoated samples (control) were analysed based on parameters such as colour change, hardness, mass loss, bacterial growth, and physical damage. The concentrations of lemongrass essential oil used were 0%, 0.5%, 1%, 1.5%, and 2% (v/v). The results showed that a concentration of 2% was the most effective in enhancing the function of

the protective coating, which was characterised by reduced weight loss, better texture retention, and inhibited microbial activity. After 20 days of storage, the fruit exhibited weight loss of 0.05% at 30 °C and 0.04% at 4 °C. In addition, the colour change was relatively small, and the tomato flavour remained stable. Thus, the use of a natural protective coating based on lemongrass essential oil can reduce post-harvest losses in perishable fruit.

Keywords: Edible film, essential oil, post-harvest, tomato

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INTRODUCTION

Tomatoes (*Solanum lycopersicum* L.) are cultivated worldwide and contain various vitamins and phytochemicals that are extremely beneficial for human health. Nevertheless, due to their climacteric nature, tomatoes face various issues throughout the post-harvest period since they ripen even after harvesting. It is caused by several physical, physiological, and pathological factors that greatly influence tomato physiology (Ozer et al., 2022). In addition, postharvest technologies such as edible coatings and modified atmosphere packaging play an important role in maintaining quality and extending shelf life by reducing moisture loss and regulating gas exchange (Garcia et al., 2014; Murugan et al., 2022; Perez-Vazquez et al., 2023). Such an interrelation of factors negatively affects the shelf life and commercial potential of the fruit (Colombié et al., 2017). Moreover, ethylene is one of the hormones that regulate ripening and is synthesised by 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) genes. Thus, extensive knowledge of the mechanisms of ethylene-based tomato ripening is of paramount importance for increasing the shelf life of tomatoes (Pontiggia et al., 2019).

To maintain the quality of the fruits throughout their transport and storage period, post-harvest processing is required. The physiological activities leading to fruit ageing, such as respiration, senescence, and ripening, can be regulated using post-harvest processing. The application of edible films, which is an effective method for enhancing the quality and shelf-life of fresh fruits, has received substantial attention in recent years. Normally used as a thin film, edible films create a barrier that inhibits the deterioration process involving activities such as respiration, oxidation, and dehydration (Duan et al., 2019). Edible protective films are an effective and sustainable way to preserve fruits and vegetables. There are three major components in edible films, including hydrocolloids (proteins and polysaccharides), lipids (fatty acids), and composites/hydrocolloids-lipid blends (Liyanapathirane et al., 2023). The use of this film effectively reduced oxidation reactions, slowed respiration, and suppressed physiological disorders in horticultural products (Garcia et al., 2014). Avramescu et al. (2020) stated that edible protective films generally consist of high-quality food ingredients, thus maintaining the nutritional quality and sensory characteristics of fresh produce. Meanwhile, Ruan et al. (2019) explained that polysaccharide-based films have excellent barrier properties due to the presence of tightly arranged hydrogen bonds within their structure.

Selecting an appropriate coating substrate is another crucial factor. In view of the findings presented by Khezerlou et al. (2023) and Aswini et al. (2022), the inclusion of diverse biopolymer blends, including protein-based, polysaccharide-based, and lipid-based biopolymers, might be useful in improving the performance of the films and consequently minimising the losses of moisture, gases, and oxidative reactions within the produce. The types of base biopolymers that can be used for coating have been identified by

Pacheco et al. (2021) and include pullulan, maltodextrin, starch, hydrolysed collagen, gelatin, alginate, chitosan, and pectin.

The study conducted by Siswanto et al. (2023) investigated the use of maltodextrin as a film-forming material for stabilising lemongrass oil emulsions. Their findings demonstrated that maltodextrin significantly improved the quality and stability of the film, attributed to its capacity to produce a strong yet flexible film structure. This makes it an effective coating agent because it helps reduce water losses and decrease respiration rates (Perez-Vázquez et al., 2023). Nevertheless, the application of maltodextrin as a film-forming agent in thin edible films is limited. As a natural polysaccharide, pectin contributes to the film-forming ability of the coating and enhances moisture retention, thereby preserving the freshness and texture of produce.

According to Rohasmizah and Azizah (2022), pectin is ideal for producing edible films and coatings because of its superior gelling, thickening, and stabilising characteristics. Additionally, coatings made from pectin can prolong the shelf life of fruits by as much as 11 days by slowing down changes in weight loss, total soluble solids, pH, total acidity, firmness, and colour (Menezes & Athmaselvi, 2016). Simultaneously, another study assessed the use of coatings made from pectin-maltodextrin and sodium chloride on starfruit. This involved a 1% solution of pectin-maltodextrin in a 60:40 ratio, which demonstrated promise in preserving fruit quality during storage (Mohd Suhaimi et al., 2021). Several studies have individually analysed the effects of pectin and maltodextrin in creating edible films from fruits. Together, these two ingredients have proven useful in developing edible films that can entrap and retain bioactive molecules and increase film durability (Nazari Kermanshahi et al., 2021; Slimani et al., 2025). Further investigations should be conducted to determine their optimal concentrations and processing parameters.

Natural antimicrobials and antioxidants, such as essential oils, have attracted significant research interest for their application in improving the functional attributes of edible films. Films embedded with essential oils (EOs) have proven their ability to lower microbial populations in food (Lakshan et al., 2024; Yousuf et al., 2021). In the current study, lemongrass EOs (*Cymbopogon nardus*) were incorporated into edible coatings as antimicrobial agents. The composition of lemongrass EOs includes limonene, geraniol, and citronellol, which are known for their antibacterial activity and capability to retard bacterial proliferation (Trinetta et al., 2017). Lemongrass EOs are widely known for their antibacterial, antifungal, and antiviral properties, making them effective against a wide range of pathogenic microorganisms. Active compounds, phenols, and aldehydes, inhibit microbial activity by destabilising the lipid layer and disrupting bacterial cell membranes. Furthermore, lemongrass essential oil also plays a role in preventing the formation of bacterial biofilms (Mukarram et al., 2022). Several recent investigations found that the use of lemongrass essential oils (EOs) nanoemulsions incorporated within chitosan coatings

leads to an increase in the storage period for bananas (Putra et al., 2025). It has also been found that a mixture coating, which includes xanthan gum with lemongrass EOs, can be helpful in reducing oxidative stress in Kinnow mandarin fruits, thereby improving their nutritional status over the long-term storage (Bajaj et al., 2024). Therefore, this study aimed to examine the use of maltodextrin and pectin coatings, supplemented with lemongrass essential oils, applied to tomatoes.

MATERIALS AND METHODS

Materials

Servo F1 is a fresh tomato sourced from the Bandungan Garden located in Semarang Regency, Central Java, Indonesia. Fruits were harvested at the break-ripening stage and visually selected to ensure uniformity of size and shape, and freedom from physical damage or lesions, to minimise variability in the experimental material. Maltodextrin (MD) with dextrose equivalents (DE) of 10-12, used to produce the edible coating, was provided by Ensui Sugar Refining Co., Ltd. (Tokyo, Japan). Sigma-Aldrich (Jakarta, Indonesia) purchased glycerol (Gly), Tween 80, and pectin (9000-69-5 with 485 kDa). Atsiri House in Karanganyar, Indonesia, sells lemongrass essential oil (EO) with 99% purity.

Methods

Formulation of Edible Coating Solution

Dissolve 10 g of MD in 100 mL of distilled water and heat the mixture to 75°C on a hotplate. To prepare the edible coating solution, 2 mL of Gly, Tween 80 at a ratio of 1:2 (w/v), distilled water and EO at different ratios (0, 0.5, 1, 1.5, and 2% v/v) relative to the total volume of the coating solution were added. The solution was stirred continuously at 2000 rpm for 13 minutes to obtain a homogeneous solution using an Ultra Turrax Homogeniser (IKA T10). After mixing, 3 g pectin was added to the solution, and the mixture was heated and stirred for 10 minutes to obtain a homogeneous film solution.

Edible Coating Treatment of Tomatoes

The selected tomatoes should be of the same size, colour, and free from defects. After cleaning with water, the tomatoes were immersed in a chlorine solution for two minutes, rinsed with distilled water, and air-dried at room temperature. A total of 30 mL of coating solution was applied to each basket of 25 tomatoes (1.2 mL/fruit), and a hand sprayer was used to evenly coat the tomatoes. Before use or storage, the tomatoes are dried for twenty minutes at room temperature until they are no longer sticky.

Storage Conditions

After the coating treatment, all tomato samples were stored under controlled conditions. The fruits were stored at 4 °C and 30 °C with a controlled relative humidity (RH) of 75%, regulated by a saturation salt control system, for a 20-day storage period. The effect of the edible coating on post-harvest tomato quality was assessed based on observations collected on days 0, 5, 15, and 20 of storage. The parameters evaluated included shelf life, weight loss, firmness loss, skin colour change, microbial growth, and fruit damage level.

Fruit Remaining Weight Analysis

For each treatment group, fruit weight was measured with an analytical balance on days 0, 5, 10, 15, and 20. The initial weight (W_0) and the corresponding weight at time t (W_t) were recorded at each sampling interval. The amount of fruit weight loss (%) was calculated based on the relative reduction in weight over time, as described in Equation 1.

$$\text{Fruit weight loss (\%)} = \frac{W_0 - W_t}{W_0} \times 100\% \quad [1]$$

where W_0 is the initial weight, and W_t is the weight on the day. To express the proportion of weight retained by the fruit, the remaining weight (%) was calculated as shown in Equation 2.

$$\text{Remaining weight (\%)} = 100 - \text{fruit weight loss (\%)} \quad [2]$$

Fruit Firmness Analysis

Fruit firmness was determined using an analogue penetrometer (GY3, Y Wang Qiang, China) with a penetration depth of up to 10 mm. Measurements were taken at two different points on the fruit in each treatment group (Bejaei, 2022).

Colour Analysis

The surface colour was measured using a colourimeter (WR-10QC; FRU, China). The L^* (brightness), a^* (green-red colour intensity), and b^* (blue-yellow colour intensity) values resulting from the emission of specific wavelengths of light through the sample surface were based on the colour change of the sample. These values were obtained from the average of three readings taken approximately 120° apart to account for natural colour heterogeneity. The colour and absorbance of the sample indicate the concentration in the sample (Ruelas-Chacon et al., 2017). Before use, the WR-10QC colourimeter was calibrated using standard black and white calibration tiles. The reference values obtained were $X = 79.0$, $Y = 83.9$, and $Z = 87.9$.

Microbial Growth Analysis

A 25-gram sample was taken aseptically with a sterile scalpel and placed into a sterile container for microbiological analysis. Then, 225 mL of Butterfield phosphate buffer solution was added, and the mixture was homogenised for 2 minutes to achieve a 10^{-1} dilution. For the 10^{-2} dilution, 1 mL of homogenate was added to 9 mL of Butterfield phosphate buffer solution, and the next dilution (10^{-3}) was prepared by adding 1 mL of the 10^{-2} dilution and adding it to 9 mL of Butterfield phosphate buffer solution. For each dilution (10^{-1} , 10^{-2} , and 10^{-3}), one millilitre was pipetted into sterile Petri dishes in duplicate. Next, 15 mL was added to each plate for plate count (PCA) at 45 °C. To ensure proper mixing, the dish was shaken from side to side. The dish was stored in an inverted position in an incubator for 48 hours at 35 °C after solidification. A control was prepared without the sample by mixing the diluent with PCA (Siburian et al., 2021).

Fruit Damage Intensity Analysis

Subjective parameters were used to evaluate the degree of fruit damage. Observations were made by applying the damage intensity formula to each tomato fruit. The damage level was measured from 0 to 25%. Fruits with more than 25% damage were considered to have no marketable value and were removed from treatment. To classify fruit damage, the following grading scale was used: (0) no symptoms; (1) 1-5%; (2) 6-10%; (3) 11-15%; (4) 16-20%; and (5) 20-25%. Grading criteria included the presence of brown or black spots on the fruit surface, foul-smelling microbial infection, and severe softening due to internal tissue damage. Furthermore, the intensity of fruit damage can be calculated using the formula proposed by Townsend and Heuberger (1967), as cited by Kremer and Unterstenhofer (1967), as presented in Equation 3.

$$\text{Intensity of fruit damage (\%)} = \frac{\sum(n \times v)}{N \times V} \times 100 \quad [3]$$

where n is the number of fruits in each rating, v is the fruit damage scoring value, N is the total number of fruits used in one experimental unit, and V is the maximum score (5).

Statistical Analysis

SPSS Statistics 25 (IBM, USA) was used to statistically analyse the fruit quality measurement data. To ensure the reliability of the results, each measurement was performed three times ($n = 3$). To compare means and standard deviations (SD) at the significance level, Duncan's Multiple Range Test (DMRT) was used, with $p < 0.05$.

RESULTS AND DISCUSSION

The Impact of Incorporating Lemongrass Essential Oils into Maltodextrin-pectin Edible Coatings on the Weight Loss of Tomatoes

Physiological weight loss is one of the primary and prominent disorders of fresh produce and is caused by transpiration and respiration. Consistent weight loss was observed in tomato fruits during the storage period in all treatments. This may result from the physiological adaptation of tomato fruits to storage conditions and the optimisation of the coating effect after multiple applications. The weight loss of fruit coated with lemongrass essential oil is due to the oil layer acting as a barrier on the fruit's surface. The lipid layer reduces permeability to water vapour and respiratory gases, thus slowing the rate of mass loss due to transpiration. By inhibiting diffusion at the cuticle-air interface, this layer maintains water balance within the fruit. Although bioactive components can influence metabolic processes, weight loss is primarily due to the physical effects of the oil layer, which suppresses moisture loss. Figure 1 shows the effect of a maltodextrin-pectin-based edible coating with lemongrass essential oil on tomato weight loss. All samples exhibited gradual weight loss during storage. At 30 °C and 4 °C, all treatments showed significant weight loss, which began to appear on the fifth day after coating. Over the 20 days, weight loss was most pronounced during the ripening stage and storage. Tomatoes lose weight because of diminished synthesis before they are harvested (Shehata et al., 2021).

This was due to the continuous breakdown of carbohydrates into glucose and fructose. These are then converted to CO₂ and H₂O, which evaporate if they are not replaced. The application of an edible protective coating containing maltodextrin, pectin, and 2% lemongrass essential oil resulted in the lowest weight loss; the control treatment showed the highest weight loss, reaching 0.14% at 30 °C and 0.11% at 4 °C after 20 days.

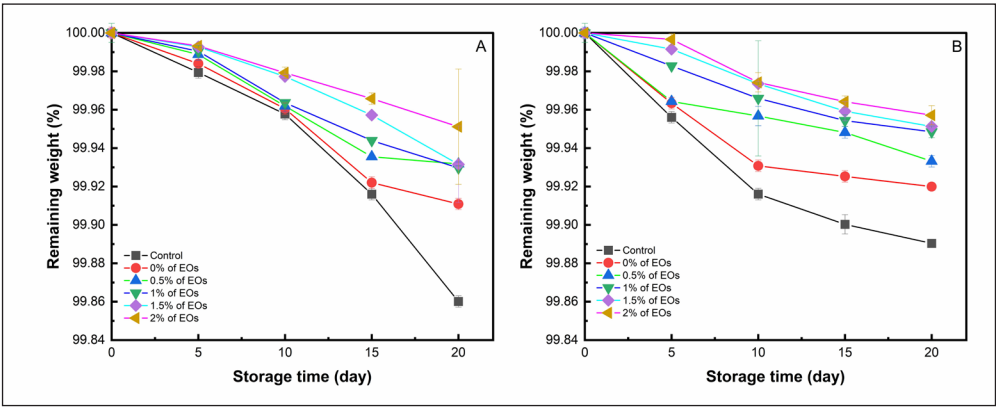


Figure 1. Effect of different concentrations of lemongrass EOs on the remaining weight (%) of tomatoes coated with maltodextrin-pectin during storage at (A) 30° C and (B) 4 °C. Points represent the means, and error bars show SD

This is because of the hydrophobic nature of lemongrass essential oil, which restricts the movement of water vapour, and its antimicrobial nature, which inhibits the growth of fungi and bacteria (Praseptianga et al., 2017).

Differences in the rate of weight loss at various storage temperatures indicated that a low temperature of 4 °C was more effective in reducing the shrinkage process in tomatoes. The application of an edible coating consisting of maltodextrin-pectin and lemongrass essential oil to tomatoes during cold storage prevents them from losing weight. This is due to respiration and transpiration, which remove substrate and water from the body. In a study conducted by Sucheta et al. (2019), an edible protective film made from commercial pectin, cornstarch, and beetroot powder reduced oxygen (O₂) levels during the first 15 days of storage. Carbon dioxide (CO₂) concentrations increased between days 15 and 20 of storage. According to Fagundes et al. (2015), a similar trend was observed in cherry tomatoes stored at low temperatures. This suggests that storing fresh fruit in a climate with a film coating can affect oxygen and carbon dioxide concentrations within the fruit while maintaining high water content. Due to the faster water loss in uncoated fruit, stress on the fruit can be reduced.

Effect of Incorporating Lemongrass EOs into Maltodextrin-pectin Edible Coatings on the Firmness of Tomatoes

Although both coated and uncoated tomatoes showed a decrease in firmness during storage, uncoated fruits showed a significant decrease in firmness ($p < 0.05$) within 20 days of storage (Figure 2). This can be attributed to rapid ripening, which results in rapid softening. According to Ali et al. (2010), the decrease in firmness as fruit ripening progresses is caused by the degradation of cell structure, cell wall composition, and intracellular materials.

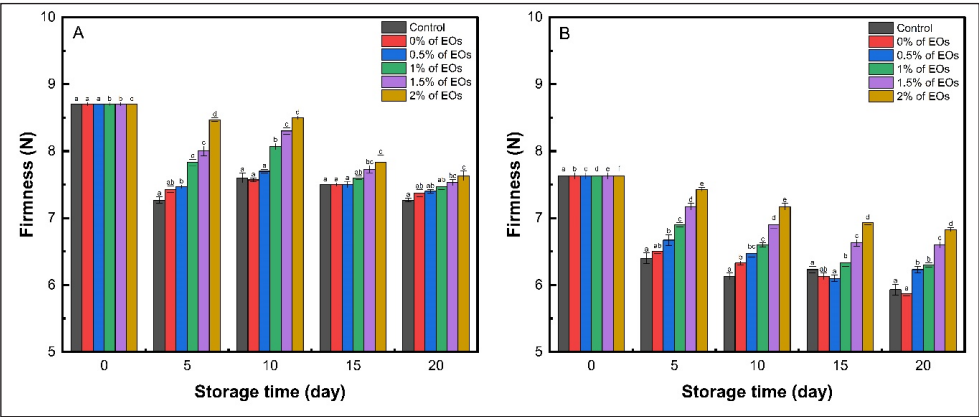


Figure 2. Effect of different concentrations of lemongrass EOs on the firmness of tomatoes coated with maltodextrin-pectin during storage at (A) 30 °C and (B) 4 °C. The values are presented as mean $\pm$ standard deviation of three replicates ($n = 3$). Means with different lowercase superscripts within the same column indicate significant differences between the film samples ($p < 0.05$)

This study showed that the firmness of tomatoes coated with an edible protective coating and infused with lemongrass essential oil significantly affected their quality during storage. However, this effect was not observed in the control group. Although the control group did not show a significant difference, Duncan's test indicated otherwise. It was determined that the main factor contributing to the daily changes in tomato samples was damage. The data showed an increase in firmness from five to ten days of storage, followed by a decrease until the end of the storage period. The addition of lemongrass essential oil resulted in a significant change from the first to the fifth day. However, treatments with 1% and 1.5% lemongrass essential oil did not differ from the treatment with 2% lemongrass essential oil. The results demonstrated that at both 30 °C and 4 °C, the samples without the edible coating underwent the most significant changes in firmness compared to those with the coating. Edible coatings that minimise weight loss are more effective in preserving fruit texture, particularly firmness, which is closely linked to the water content (Mohd Suhaimi et al., 2021). Perdana et al. (2021) reported that the addition of EOs to film-forming solutions can reduce water vapour permeability. The reduction in water vapour permeability is associated with the hydrophobic properties of the edible coating. The addition of EOs enhanced the hydrophobic nature of the coating, resulting in lower respiration and transpiration rates, thereby extending the shelf life of the fruit. This study found that adding lemongrass EOs caused less textural alteration in tomatoes, especially when higher concentrations of the EOs were used.

Effect of Incorporating Lemongrass EOs into Maltodextrin-pectin Edible Coatings on the Microbial Growth in Tomatoes

Microorganisms such as bacteria, moulds, and yeasts are the primary culprits behind food spoilage, particularly in perishable items with a limited shelf life after harvest. If fruit becomes contaminated and microorganisms grow in it, this can cause a number of changes, such as unpleasant odours, shifts in taste, and unexpected colour changes.

Figure 3 shows a clear difference in microbial growth on tomatoes coated with maltodextrin-based edible coatings and pectin enriched with lemongrass essential oil during 20 days of storage at 30 °C and 4 °C. Microbial growth was faster at 30 °C than at 4 °C because environmental factors significantly influenced microbial activity. A temperature of 30 °C is within the optimal range for the growth of mesophilic microorganisms on fruits, such as bacteria and fungi that cause decay (Perdana et al., 2021). This situation causes microbial activity to increase rapidly, thus accelerating cell growth and the colonisation process on the fruit surface. Furthermore, high storage temperatures accelerate ripening and senescence in tomatoes, weakening their cell structure and increasing nutrient availability for microorganisms (Joaquina et al., 2013). This creates an environment which favors the growth and metabolism of microorganisms.

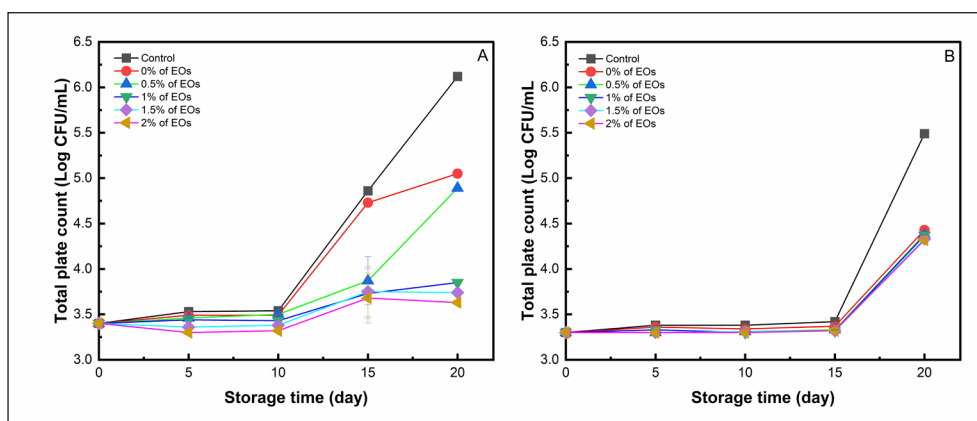


Figure 3. Effect of different concentrations of lemongrass EOs on the microbial growth profile on tomatoes coated with maltodextrin-pectin during storage at (A) 30 °C and (B) 4 °C

Alternatively, 4 °C is lower than the ideal temperature for the growth of microbes causing spoilage; therefore, it inhibits microbial growth and metabolism. These results support the findings by Fagundes et al. (2015) that low temperatures have better results in hindering the microbial growth on fruits. The control group displayed greater microbial growth at both temperatures. Thus, it was clear that adding lemongrass essential oils to maltodextrin-pectin edible coatings has inhibitory effects on microbial growth in fruits. Greater amounts of the lemongrass essential oil at higher concentrations (e.g., 1%, 1.5%, and 2%) at 30 °C and 4 °C had a better inhibitory effect on microorganisms compared to other groups. This result was attributed to the presence of certain compounds within lemongrass EOs that inhibit microbial growth in fruits.

Some of the important chemical components in lemongrass EO include citronellol, d-limonene, and lemongrass, which can inhibit the growth of *Listeria monocytogenes* bacteria in fruits by killing microorganisms (Faheem et al., 2022). Microorganism activity was inhibited using lemongrass EOs on edible films based on maltodextrin-pectin at high concentrations. Some components of EOs are more effective in inhibiting microorganisms if used in high amounts (Perdana et al., 2021).

Conversely, the reduction in visible microbial symptoms, such as the appearance of a foul odour and the spread of lesions, is likely due to the natural bioactivity of lemongrass essential oil. The monoterpene content of this oil, particularly its citral-rich component, provides antimicrobial and antioxidant properties. Research findings indicate that these characteristics inhibit surface colonisation and slow lesion development, thus complementing the moisture-reducing effect of the oil films. The more targeted and sustained inhibition of microbial growth, exceeding the effect of reducing water loss, suggests the involvement of chemical mechanisms in addition to the physical function of the films.

Effect of Incorporating Lemongrass EOs into Maltodextrin-pectin Edible Coatings on the Colour Change of Tomatoes

Colour is one of the primary sensory attributes of fresh fruits and vegetables, and significantly influences consumer acceptance. Furthermore, colour serves as an important indicator for assessing quality during storage and can be used to monitor the degradation and ageing processes in food products. Table 1 presents changes in tomato colour values based on the colourimetric parameters L^* , a^* , and b^* . The findings show that the brightness level (L^*) of the tomato fruit contributes to the conservation process during 20 days of storage at a temperature of 30 °C. There was a significant difference in the levels of L^* brightness among treatments with different concentrations of lemongrass essential oils (0, 0.5, 1, 1.5, and 2%). The control treatment exhibited the highest decrease in lightness, showing that the tomato fruit became darker. Analysis of the a^* colour test for tomatoes using edible coatings showed considerable variations in the a^* value during storage. The tomato fruits treated with 0%, 0.5%, and 1% lemongrass EOs recorded higher a^* values compared to the control group on day five. However, on day 10, samples with 1% lemongrass EOs had increased in a^* , and those with 0% and 0.5% lemongrass EOs also registered increases in their a^* values. The yellowness and blueness of tomato fruits were found to be significantly affected by various treatments after 20 days of storage at 30 °C. Significant variations were noted among 0%, 1.5%, and 2% lemongrass EOs treatments.

Observations of the colour of tomatoes stored at 4 °C showed a decrease in brightness (L^*) and a shift in colour value toward blue-yellow (b^*) with storage duration. Horticultural products, particularly tropical fruits, are generally susceptible to low temperatures. At cold temperatures, some metabolic processes slow down and can even stop if the temperature exceeds a critical limit that disrupts metabolic balance. This condition can lead to the limitation of molecules that are important for certain reactions and the accumulation of harmful compounds, which ultimately triggers cell damage, such as the appearance of brown spots. Polyphenol oxidase (PPO) contributes to this process by converting phenolic compounds into quinones, which then form brown pigments (Ferreira Holderbaum et al., 2010).

From the results obtained, it is clear that the edible coating made up of maltodextrin and pectin, in combination with different amounts of lemongrass essential oils, resulted in a more prolonged colour change compared to the control treatment of the edible coating alone. Respiration is also a factor in tomato fruit colour change. Tomatoes with inhibited respiration rates tend to experience minimal colour change due to the surface layer, whereas more pronounced colour changes occur when respiration is increased. The use of an edible coating made from maltodextrin-pectin with the addition of 2% lemongrass essential oil has been shown to cause only minor colour changes in tomatoes. This coating forms a semi-permeable barrier on the fruit's surface that can limit gas exchange, suppress respiration rates, and slow the ripening process.

Table 1
Changes in colour parameters of tomatoes with different concentrations of lemongrass EOs incorporated into maltodextrin-pectin edible coating during storage at 30 °C and 4 °C

Treatment	Storage at 30 °C				
	0 day	5 day	10 day	15 day	20 day
Colour Parameter: L^*					
Control	49.34 ^a ± 0.06	44.78 ^a ± 0.19	37.41 ^a ± 0.20	37.42 ^a ± 0.16	35.31 ^a ± 0.18
0% of EOs	45.13 ^b ± 0.11	44.13 ^a ± 0.01	43.45 ^b ± 0.14	40.00 ^a ± 0.39	35.12 ^a ± 0.29
0.5% of EOs	47.04 ^{ab} ± 0.01	45.47 ^a ± 0.12	40.93 ^{ab} ± 0.02	38.79 ^a ± 0.06	38.32 ^a ± 0.24
1% of EOs	46.52 ^{ab} ± 0.05	46.38 ^a ± 0.41	41.43 ^{ab} ± 0.09	38.10 ^a ± 0.01	35.86 ^a ± 0.08
1.5% of EOs	47.43 ^{ab} ± 0.13	42.83 ^a ± 0.16	41.67 ^{ab} ± 0.20	41.51 ^a ± 0.04	39.45 ^a ± 0.10
2% of EOs	45.73 ^b ± 0.09	45.63 ^a ± 0.14	42.71 ^b ± 0.01	42.34 ^a ± 0.01	41.51 ^a ± 0.06
Colour Parameter: a^*					
Control	3.21 ^c ± 0.04	6.40 ^b ± 0.03	6.42 ^b ± 0.03	6.45 ^b ± 0.02	9.34 ^c ± 0.16
0% of EOs	4.71 ^d ± 0.01	5.65 ^c ± 0.00	6.04 ^b ± 0.21	6.05 ^b ± 0.20	7.35 ^d ± 0.03
0.5% of EOs	3.12 ^a ± 0.01	3.22 ^a ± 0.00	3.66 ^a ± 0.41	3.74 ^a ± 0.43	6.68 ^a ± 0.23
1% of EOs	2.67 ^c ± 0.01	3.14 ^d ± 0.08	4.19 ^c ± 0.20	4.20 ^c ± 0.20	5.15 ^c ± 0.00
1.5% of EOs	2.78 ^b ± 0.06	3.33 ^a ± 0.09	3.37 ^a ± 0.07	3.47 ^a ± 0.02	4.12 ^b ± 0.16
2% of EOs	2.57 ^f ± 0.01	2.87 ^e ± 0.02	3.77 ^d ± 0.00	3.77 ^d ± 0.00	3.84 ^f ± 0.02
Colour Parameter: b^*					
Control	19.72 ^a ± 0.15	19.06 ^a ± 0.29	14.35 ^a ± 0.04	12.50 ^a ± 0.02	12.36 ^a ± 0.02
0% of EOs	21.07 ^b ± 0.04	20.53 ^b ± 0.07	19.99 ^b ± 0.40	12.47 ^a ± 0.02	11.73 ^a ± 0.12
0.5% of EOs	19.58 ^a ± 0.07	17.16 ^c ± 0.01	17.01 ^c ± 0.27	13.95 ^b ± 0.08	12.99 ^b ± 0.03
1% of EOs	19.36 ^a ± 0.11	17.83 ^c ± 0.15	16.05 ^c ± 0.04	14.67 ^b ± 0.01	9.30 ^c ± 0.01
1.5% of EOs	21.88 ^c ± 0.25	21.60 ^d ± 0.05	16.82 ^c ± 0.19	15.77 ^c ± 0.03	9.96 ^c ± 0.03
2% of EOs	20.94 ^b ± 0.03	20.63 ^b ± 0.06	17.56 ^c ± 0.07	13.53 ^b ± 0.07	7.31 ^d ± 0.05
Treatment	Storage at 4 °C				
	0 day	5 day	10 day	15 day	20 day
Colour Parameter: L^*					
Control	39.08 ^a ± 0.04	38.42 ^a ± 0.14	38.23 ^a ± 0.00	34.68 ^a ± 0.13	33.64 ^a ± 0.11
0% of EOs	39.12 ^a ± 0.04	38.90 ^a ± 0.01	38.42 ^a ± 0.37	36.22 ^a ± 0.08	35.63 ^a ± 0.00
0.5% of EOs	36.25 ^b ± 0.18	34.36 ^b ± 0.06	33.48 ^b ± 0.00	33.44 ^b ± 0.00	32.43 ^b ± 0.10
1% of EOs	38.84 ^a ± 0.00	35.16 ^b ± 0.00	34.90 ^b ± 0.05	33.75 ^b ± 0.00	33.40 ^b ± 0.10
1.5% of EOs	35.36 ^b ± 0.02	35.30 ^b ± 0.13	34.76 ^b ± 0.01	34.09 ^b ± 0.00	32.85 ^b ± 0.09
2% of EOs	35.47 ^b ± 0.01	35.30 ^b ± 0.10	34.50 ^b ± 0.00	32.61 ^b ± 0.00	32.57 ^b ± 0.03
Colour Parameter: a^*					
Control	6.68 ^b ± 0.23	3.74 ^c ± 0.43	3.66 ^c ± 0.41	3.22 ^c ± 0.00	3.12 ^c ± 0.01
0% of EOs	4.28 ^c ± 0.16	3.47 ^c ± 0.02	3.37 ^c ± 0.07	3.33 ^c ± 0.09	3.21 ^c ± 0.01
0.5% of EOs	4.12 ^c ± 0.16	3.47 ^c ± 0.02	3.37 ^c ± 0.07	3.33 ^c ± 0.09	3.21 ^c ± 0.01
1% of EOs	3.94 ^c ± 0.16	6.45 ^{ab} ± 0.02	6.42 ^{ab} ± 0.03	6.40 ^{ab} ± 0.03	6.68 ^{ab} ± 0.04
1.5% of EOs	7.35 ^{ab} ± 0.03	6.06 ^{abc} ± 0.20	6.04 ^{abc} ± 0.21	5.65 ^{abc} ± 0.00	4.71 ^{abc} ± 0.01
2% of EOs	5.55 ^{bc} ± 0.16	4.00 ^{bc} ± 0.20	3.77 ^c ± 0.00	2.87 ^c ± 0.02	3.84 ^c ± 0.01

Table 1 (continued)

Treatment	Storage at 30 °C				
	0 day	5 day	10 day	15 day	20 day
Colour Parameter: <i>b</i>*					
Control	22.92 ^a ± 0.62	17.84 ^c ± 0.62	15.47 ^d ± 0.62	15.25 ^d ± 0.62	15.10 ^d ± 0.62
0% of EOs	16.42 ^c ± 0.60	16.50 ^c ± 0.60	15.76 ^c ± 0.60	14.35 ^e ± 0.60	13.25 ^e ± 0.60
0.5% of EOs	28.71 ^b ± 1.00	19.46 ^c ± 1.00	19.23 ^c ± 1.00	19.08 ^c ± 1.00	14.67 ^e ± 1.00
1% of EOs	14.96 ^f ± 0.60	17.10 ^d ± 0.60	17.05 ^d ± 0.60	17.03 ^d ± 0.60	14.90 ^d ± 0.60
1.5% of EOs	17.11 ^d ± 0.60	14.94 ^f ± 0.60	14.81 ^f ± 0.60	14.50 ^f ± 0.60	14.35 ^f ± 0.60
2% of EOs	14.55 ^g ± 0.60	14.39 ^g ± 0.60	13.13 ^g ± 0.60	11.00 ^g ± 0.60	9.22 ^g ± 0.60

Note. *Values are presented as mean ± standard deviation from three replicates (n = 3). Means with different lowercase superscripts within the same column indicate significant differences between the film samples (p < 0.05)

As a polysaccharide-based coating, maltodextrin-pectin forms a hydrocolloid matrix that inhibits air movement between the fruit and the external environment. This condition changes the internal environment of the fruit, delaying respiration and ripening processes. Furthermore, the combination of hydrocolloids and lipids produces a coating that is effective in reducing respiration rates while reducing water loss.

The use of an edible coating made of maltodextrin-pectin with lemongrass EOs has proven to be highly effective in terms of preventing the colour change in tomatoes as compared to non-coated control products. It seems that better colour preservation and lesser tissue destruction can be attributed to a combined effect of the oil film and the biological actions of lemongrass EOs. The oil coating acts as a barrier against oxygen transport and moisture stress, thus inhibiting the oxidation process and preventing tissue softening. At the same time, the antioxidants present in lemongrass EOs can slow down oxidising processes leading to pigment and membrane destruction, while the antimicrobial activities can prevent lesion formation. Collectively, these synergistic mechanisms minimise discolouration and decrease tissue damage during storage. The oil layer inhibits degradation caused by oxidation and moisture, whereas the antioxidant and antimicrobial properties of lemongrass essential oil help maintain cell integrity and prevent lesion development.

Figure 4 depicts the physical appearance of the tomatoes after coating with the edible coating at various storage temperatures: (A) 30 °C and (B) 4 °C. At the 30 °C storage temperature, the tomato colour changed to completely red on the 20th day, suggesting that the tomato had fully matured. In contrast, the coated tomatoes showed delayed ripening, with increased effectiveness observed at higher concentrations of lemongrass essential oil, as evidenced by the persistence of a yellowish colour at the end of the storage period. The colour change under edible coating treatment at 4 °C storage temperature showed a significant difference between the control and the maltodextrin-pectin coating with lemongrass essential oil.

Control fruits changed colour from green to yellow at the end of the storage period, but the coated samples showed a clear delay in colour change during storage. Maltodextrin-pectin coating with lemongrass essential oil significantly delayed chlorophyll degradation and ripening (Figure 4). The coated fruit maintained a predominantly green-yellow colour for up to 20 days, whereas yellow to reddish colouration was observed at 30 °C. Edible coatings made of maltodextrin-pectin combined with varying concentrations of lemongrass essential oil significantly affected colour change. The storage temperature is also important for maintaining fruit colour and quality throughout the storage period. These results are consistent with research conducted by Adjouman et al. (2018), which showed that the polysaccharide layer helps limit the increase in the a^* value of tomatoes compared to the control group. This phenomenon is likely due to the layer inhibiting oxygen exchange (Olivas et al., 2007). Due to their structural properties, polysaccharides such as maltodextrin and pectin act as oxygen barriers (Fagundes et al., 2014). The respiration rate is influenced by the amount of available oxygen, so the effectiveness of this layer in limiting oxygen diffusion is crucial. Qualities such as firmness and retardation of colour change are maintained by this layer, both of which are strongly influenced by respiration (Fagundes et al., 2014). Furthermore, storing tomatoes at low temperatures is crucial for maintaining their quality because cold conditions inhibit the genes responsible for carotenoid biosynthesis, which helps maintain carotenoid content and prolong freshness (Li et al., 2021).



Figure 4. Effect of different concentrations of lemongrass EOs on the physical changes in tomatoes coated with maltodextrin-pectin before and after storage at (A) 30 °C and (B) 4 °C

Effect of Incorporating Lemongrass EOs into Maltodextrin-pectin Edible Coatings on the Intensity of Tomato Fruit Damage

Variations in the severity of damage to tomato fruits are shown in Table 2. The table highlights significant differences in the intensity of damage to tomatoes stored at 30 °C and 4 °C, as well as the impact of maltodextrin-pectin-based edible protective coatings with varying concentrations of lemongrass essential oil over a 20-day period. These findings indicate that the intensity of damage to tomato fruits is significantly influenced by storage temperature and the use of edible protective coatings. Fruit damage increased gradually during storage at 30 °C, with the control group reaching 20.4% on day 20. However, treatment using a maltodextrin-pectin coating enriched with lemongrass essential oil significantly reduced the damage level. The 2% concentration showed the best results with a damage level of only 9.9% at 4 °C, whereas the control group at that temperature recorded damage of 0.9% on day 20. In addition, throughout the storage period, the samples with the addition of 1%, 1.5%, and 2% lemongrass essential oil did not experience damage.

Low storage temperature will slow down the process of degradation and metabolism. As such, physiological decline and microbial growth will be greatly minimised (Alqahtani et al., 2025).

Table 2
Effect of different concentrations of lemongrass EOs on the intensity of damage observed in tomato fruit coated with a maltodextrin-pectin during storage at 30 °C and 4 °C

Treatment	Intensity of Fruit Damage (%)				
	Storage at 30 °C				
	0 day	5 day	10 day	15 day	20 day
Control	0 ^a ± 0.00	0.9 ^a ± 0.00	4.2 ^a ± 0.00	11.7 ^a ± 0.00	20.4 ^a ± 0.00
0% of EOs	0 ^a ± 0.00	1.5 ^b ± 0.00	4.2 ^a ± 0.00	6.0 ^b ± 0.00	19.2 ^a ± 0.00
0.5% of EOs	0 ^a ± 0.00	0.6 ^c ± 0.00	3.6 ^b ± 0.00	9.9 ^c ± 0.00	12.6 ^b ± 0.00
1% of EOs	0 ^a ± 0.00	0.6 ^c ± 0.00	3.6 ^b ± 0.00	6.0 ^b ± 0.00	12.6 ^b ± 0.00
1.5% of EOs	0 ^a ± 0.00	0.3 ^d ± 0.00	1.2 ^c ± 0.00	3.6 ^c ± 0.00	10.8 ^c ± 0.00
2% of EOs	0 ^a ± 0.00	0.3 ^d ± 0.00	1.2 ^c ± 0.00	3.6 ^c ± 0.00	9.9 ^c ± 0.00

Treatment	Intensity of fruit damage (%)				
	Storage at 4 °C				
	0 day	5 day	10 day	15 day	20 day
Control	0 ^a ± 0.00	0 ^a ± 0.00	0.6 ^a ± 0.00	0.6 ^a ± 0.00	0.9 ^a ± 0.00
0% of EOs	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0.3 ^b ± 0.00	0.3 ^b ± 0.00
0.5% of EOs	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0.3 ^b ± 0.00
1% of EOs	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00
1.5% of EOs	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00
2% of EOs	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00	0 ^a ± 0.00

Note. *Values are presented as mean ± standard deviation from three replicates (n = 3). Means with different lowercase superscripts within the same column indicate significant differences between film samples (p < 0.05)

The edible coating, made from maltodextrin and pectin, serves as both a physical and gaseous barrier to prevent water loss and gas exchange, which can cause loss of fruit quality. Lemongrass essential oil enhanced the antimicrobial properties of the protective layer, providing additional protection against microbial spoilage. The significant difference in spoilage rates between the two storage temperatures indicates that temperature influences the metabolic processes and fruit degradation. Furthermore, physiological damage and microbial spoilage in tomatoes tend to increase with storage duration. Therefore, lowering the storage temperature can extend the shelf life of tomatoes while slowing the spoilage process (Shehata et al., 2021).

Tomatoes can be kept fresh for much longer periods using a protective film. The use of active agents from lemon grass essential oil in edible protective film consisting of maltodextrin and pectin will improve fruit protection. Pectin enhances the gas barrier properties of the protective coating, whereas maltodextrin forms a thin film that acts as a physical barrier and adds mechanical strength to the protective coating (Mohd Suhaimi et al., 2021). Lemongrass essential oil, as an active component, enhances the protective coating of edible tomatoes and extends their shelf life. Lemongrass essential oil also acts as an antimicrobial agent.

CONCLUSION

An edible protective film composed of 10% maltodextrin and 3% pectin exhibited excellent stability and effectively transported lemongrass essential oil. Tomato quality during storage, including colour, weight retention, firmness, and microbial control, was improved by adding lemongrass essential oil (0-2%) to this matrix. Storage at 4 °C resulted in better preservation than storage at 30 °C. This resulted in slower ripening, reduced moisture loss and tissue damage. The barrier properties of the protective layer and the antioxidant and antimicrobial effects of lemongrass essential oil caused this shrinkage. A 2% lemongrass essential oil formulation has been shown to increase preservation effectiveness and maintain fruit quality during storage. The results showed that a maltodextrin-pectin-based coating enriched with lemongrass essential oil could extend the shelf life of tomatoes. For application to various commodities, further research should focus on optimising the material composition, analysing sensory impacts, and evaluating its performance.

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REFERENCES

- Adjouman, Y. D., Nindjin, C., Kouassi, K. N., Tetchi, F. A., Amani, G. G., & Sindic, M. (2018). Effect of edible coating based on improved cassava starch on post-harvest quality of fresh tomatoes (*Solanum lycopersicum* L.). *International Journal of Nutritional Science and Food Technology*, 4(1), 1-10. <https://hdl.handle.net/2268/226474>
- Ali, A., Maqbool, M., Ramachandran, S., & Alderson, P. G. (2010). Gum arabic as a novel edible coating for enhancing shelf-life and improving postharvest quality of tomato (*Solanum lycopersicum* L.) fruit. *Postharvest Biology and Technology*, 58(1), 42-47. <https://doi.org/10.1016/j.postharvbio.2010.05.005>
- Alqahtani, N. K., Alkhamis, B., Alnemr, T. M., & Mohammed, M. (2025). Combined influences of edible coating and storage conditions on the quality of fresh dates: An investigation and predictive analysis using artificial neural networks. *Heliyon*, 11(4), Article e42373. <https://doi.org/10.1016/j.heliyon.2025.e42373>
- Aswini, M., Thahira, B. A., & Shanthana L. D. (2022). Edible coatings to enhance shelf life of fruits and vegetables: A mini-review. *Nutrition and Food Science*, 18(6), 525-538. <https://doi.org/10.2174/1573401318666220303161527>
- Avramescu, S. M., Butean, C., Popa, C. V., Ortan, A., Moraru, I., & Temocico, G. (2020). Edible and functionalised films/coatings—performances and perspectives. In *Coatings* (Vol. 10). MDPI AG. <https://doi.org/10.3390/coatings10070687>
- Bajaj, K., Kumar, A., Gill, P. P. S., Jawandha, S. K., & Kaur, N. (2024). Xanthan gum coatings augmented with lemongrass oil preserve postharvest quality and antioxidant defence system of Kinnow fruit under low-temperature storage. *International Journal of Biological Macromolecules*, 262, Article 129776. <https://doi.org/10.1016/j.ijbiomac.2024.129776>
- Bejaei, M. (2022). Converting apple textural parameters obtained from penetrometers and their relationships with sensory attributes. *Horticulturae*, 8(3), Article 269. <https://doi.org/10.3390/horticulturae8030269>
- Colombié, S., Beauvoit, B., Nazaret, C., Bénard, C., Vercambre, G., Le Gall, S., Biais, B., Cabasson, C., Maucourt, M., Bernillon, S., Moing, A., Dieuaide-Noubhani, M., Mazat, J. P., & Gibon, Y. (2017). Respiration climacteric in tomato fruits elucidated by constraint-based modelling. *New Phytologist*, 213(4), 1726-1739. <https://doi.org/10.1111/nph.14301>
- Duan, C., Meng, X., Meng, J., Khan, M. I. H., Dai, L., Khan, A., An, X., Zhang, J., Huq, T., & Ni, Y. (2019). Chitosan as a preservative for fruits and vegetables: A review on chemistry and antimicrobial properties. *Journal of Bioresources and Bioproducts*, 4(1), 11-21. <https://doi.org/10.21967/jbb.v4i1.189>
- Fagundes, C., Moraes, K., Pérez-Gago, M. B., Palou, L., Maraschin, M., & Monteiro, A. R. (2015). Effect of active modified atmosphere and cold storage on the postharvest quality of cherry tomatoes. *Postharvest Biology and Technology*, 109, 73-81. <https://doi.org/10.1016/j.postharvbio.2015.05.017>
- Fagundes, C., Palou, L., Monteiro, A. R., & Pérez-Gago, M. B. (2014). Effect of antifungal hydroxypropyl methylcellulose-beeswax edible coatings on grey mould development and quality attributes of cold-stored cherry tomato fruit. *Postharvest Biology and Technology*, 1-8. <https://doi.org/10.1016/j.postharvbio.2014.01.006>
- Faheem, F., Liu, Z. W., Rabail, R., Haq, I. U., Gul, M., Bryła, M., Roszko, M., Kieliszek, M., Din, A., & Aadil, R. M. (2022). Uncovering the industrial potentials of lemongrass essential oil as a food preservative: A review. *Antioxidants*, 11(4). <https://doi.org/10.3390/antiox11040720>

- Ferreira Holderbaum, D., Kon, T., Kudo, T., & Pedro Guerra, M. (2010). Enzymatic browning, polyphenol oxidase activity, and polyphenols in four apple cultivars: Dynamics during fruit development. *American Society for Horticultural Science*, 45(8), 1150-1154. <https://doi.org/10.21273/HORTSCI.45.8.1150>
- Garcia, C. C., Caetano, L. C., de Souza Silva, K., & Mauro, M. A. (2014). Influence of edible coating on the drying and quality of papaya (*Carica papaya*). *Food and Bioprocess Technology*, 7, 2828-2839. <https://doi.org/10.1007/s11947-014-1350-6>
- Joaquina, P., Carla, A., Marta, A., Gonçalves Elsa M., & Silva Cristina L. M. (2013). Kinetics of changes in the physical quality parameters of fresh tomato fruits (*Solanum lycopersicum*, cv. 'Zinac') during storage. *Journal of Food Engineering*, 114, 338-345. <https://doi.org/10.1016/j.jfoodeng.2012.08.024>
- Khezerlou, A., Zolfaghari, H., Forghani, S., Abedi-Firoozjah, R., Alizadeh Sani, M., Negahdari, B., Jalalvand, M., Ehsani, A., & McClements, D. J. (2023). Combining non-thermal processing techniques with edible coating materials: An innovative approach to food preservation. *Coatings*, 13(5), Article 830. <https://doi.org/10.3390/coatings13050830>
- Kremer, Fr., & Unterstenhofer, G. (1967). De l'emploi de la méthode de Townsend et Heuberger dans l'interprétation des résultats d'essais phytosanitaires [On the use of the Townsend and Heuberger method in the interpretation of the results of phytosanitary trials]. *Pflanzenschutz-Nachrichten*, 4, 625-628.
- Lakshan, N. D., Senanayake, C. M., Liyanage, T., & Lankanayaka, A. (2024). Clove essential oil emulsions-loaded arrowroot starch-beeswax-based edible coating extends the shelf life and preserves the postharvest quality of fresh tomatoes (*Solanum lycopersicum* L.) stored at room temperature. *Sustainable Food Technology*, 2, 1052-1068. <https://doi.org/10.1039/D4FB00033A>
- Murugan, A., Banu, A. T., & Lakshmi, D. S. (2022). Edible Coatings to Enhance Shelf Life of Fruits and Vegetables: A Mini-Review. *Current Nutrition & Food Science*, 18(6), 525-538. <https://doi.org/10.2174/1573401318666220303161527>
- Li, N., Wu, X., Zhuang, W., Xia, L., Chen, Y., Wu, C., Rao, Z., Du, L., Zhao, R., Yi, M., Wan, Q., & Zhou, Y. (2021). Tomato and lycopene and multiple health outcomes: Umbrella review. *Food Chemistry*, 343, 1-8, Article 128396. <https://doi.org/10.1016/j.foodchem.2020.128396>
- Liyanapathirana, A., Dassanayake, R. S., Gamage, A., Karri, R. R., Manamperi, A., Evon, P., Jayakodi, Y., Madhujith, T., & Merah, O. (2023). Recent developments in edible films and coatings for fruits and vegetables. *Coatings*, 13(7), Article 1177. <https://doi.org/10.3390/coatings13071177>
- Menezes, J., & Athmaselvi, K. A. (2016). Study on effect of pectin based edible coating on the shelf life of Sapota fruits. *Biosciences Biotechnology Research Asia*, 13(2), 1195-1199. <https://doi.org/10.13005/bbra/2152>
- Mohd Suhaimi, N. I., Mat Ropi, A. A., & Shahrudin, S. (2021). Safety and quality preservation of starfruit (*Averrhoa carambola*) at ambient shelf life using synergistic pectin-maltodextrin-sodium chloride edible coating. *Heliyon*, 7(2), Article e06279. <https://doi.org/10.1016/j.heliyon.2021.e06279>
- Mukarram, M., Choudhary, S., Khan, M. A., Poltronieri, P., Khan, M. M. A., Ali, J., Kurjak, D., & Shahid, M. (2022). Lemongrass essential oil components with antimicrobial and anticancer activities. *Antioxidants*, 11, Article 20. <https://doi.org/10.3390/antiox11010020>
- Nazari Kermanshahi, S., Sharifan, A., & Yousefi, S. (2021). Physicochemical, microstructural, bioactivity and viability characteristics of probiotic spray-dried raisin powder. *Journal of Food Measurement and Characterisation*, 15, 633-642. <https://doi.org/10.1007/s11694-020-00662-3>

- Olivas, G. I., Mattinson, D. S., & Barbosa-Cánovas, G. V. (2007). Alginate coatings for preservation of minimally processed “Gala” apples. *Postharvest Biology and Technology*, 45(1), 89-96. <https://doi.org/10.1016/j.postharvbio.2006.11.018>
- Ozer, H., Yilmaz, C., & Ozturk, B. (2022). The influence of cultivation system and modified atmosphere packaging on quality attributes of tomato fruit during cold storage. *Journal for Sustainable Production Systems*, 38(4), 258-265. <https://doi.org/10.1080/01448765.2022.2074890>
- Pacheco, M. S., Barbieri, D., da Silva, C. F., & de Moraes, M. A. (2021). A review on orally disintegrating films (ODFs) made from natural polymers such as pullulan, maltodextrin, starch, and others. *International Journal of Biological Macromolecules*, 178, 504-513. <https://doi.org/10.1016/j.ijbiomac.2021.02.180>
- Perdana, M. I., Ruamcharoen, J., Panphon, S., & Leelakriangsak, M. (2021). Antimicrobial activity and physical properties of starch/chitosan film incorporated with lemongrass essential oil and its application. *LWT*, 141, 1-8, Article 110934. <https://doi.org/10.1016/j.lwt.2021.110934>
- Perez-Vazquez, A., Barciela, P., Carpena, M., & Prieto, M. A. (2023). Edible coatings as a natural packaging system to improve fruit and vegetable shelf life and quality. *Foods*, 12, Article 3570. <https://doi.org/10.3390/foods12193570>
- Pontiggia, D., Spinelli, F., Fabbri, C., Licursi, V., Negri, R., De Lorenzo, G., & Mattei, B. (2019). Changes in the microsomal proteome of tomato fruit during ripening. *Scientific Reports*, 9(1), Article 13583. <https://doi.org/10.1038/s41598-019-50575-5>
- Praseptiangga, D., Utami, R., Khasanah, L. U., Evirananda, I. P., & Kawiji. (2017). Effect of cassava starch-based edible coating incorporated with lemongrass essential oil on the quality of papaya MJ9. *IOP Conference Series: Materials Science and Engineering*, 176(1), Article 012054. <https://doi.org/10.1088/1757-899X/176/1/012054>
- Putra, P. S. U., Adhika, D. R., Genecya, G., Al Madanie, M. S., & Asri, L. A. T. W. (2025). Evaluation of chitosan-encapsulated lemongrass (*Cymbopogon citratus*) essential oil nanoemulsion for fruit edible coating. *OpenNano*, 24, Article 100246. <https://doi.org/10.1016/j.onano.2025.100246>
- Rohasmizah, H., & Azizah, M. (2022). Pectin-based edible coatings and nanoemulsion for the preservation of fruits and vegetables: A review. *Applied Food Research*, 2(2), 1-9, Article 100221. <https://doi.org/10.1016/j.afres.2022.100221>
- Ruan, C., Zhang, Y., Sun, Y., Gao, X., Xiong, G., & Liang, J. (2019). Effect of sodium alginate and carboxymethyl cellulose edible coating with epigallocatechin gallate on quality and shelf life of fresh pork. *International Journal of Biological Macromolecules*, 141, 178-184. <https://doi.org/10.1016/j.ijbiomac.2019.08.247>
- Ruelas-Chacon, X., Contreras-Esquivel, J. C., Montañez, J., Aguilera-Carbo, A. F., Reyes-Vega, M. L., Peralta-Rodriguez, R. D., & Sanchéz-Brambila, G. (2017). Guar gum as an edible coating for enhancing shelf-life and improving postharvest quality of Roma tomato (*Solanum lycopersicum* L.). *Journal of Food Quality*, 2017, Article 8608304. <https://doi.org/10.1155/2017/8608304>
- Shehata, S. A., Abdelrahman, S. Z., Megahed, M. M. A., Abdeldaym, E. A., El-Mogy, M. M., & Abdelgawad, K. F. (2021). Extending shelf life and maintaining quality of tomato fruit by calcium chloride, hydrogen peroxide, chitosan, and ozonated water. *Horticulturae*, 7(9), Article 309. <https://doi.org/10.3390/horticulturae7090309>

- Siburian, P. W., Falah, Moh. A. F., & Mangunwikarta, J. (2021). Alginate-based edible coatings enriched with cinnamon essential oil extend storability and maintain the quality of strawberries under tropical condition. *Planta Tropika: Jurnal Agrosains (Journal of Agro Science)*, 9(1), 58-70. <https://doi.org/10.18196/pt.v9i1.10368>
- Siswanto, P. S., Ariyanto, H. D., Yulianto, M. E., Faisal, M. M., Wrdani, O. K., & Kuvshinov, D. (2023). Formulation and physical properties of citronella oil emulsion on differences in emulsifiers with the addition of maltodextrin. *Materials Today: Proceedings*, 87, 147-151. <https://doi.org/10.1016/j.matpr.2023.02.389>
- Slimani, S., Lamia, K., Akretche-Kelfat, S., Amira, O., & Hesarinejad, M. A. (2025). Enhanced antioxidant properties in functional ice cream through encapsulation of pulsed electric field-assisted *Artemisia campestris* extract. *Food Science and Nutrition*, 13, Article 70306. <https://doi.org/10.1002/fsn3.70306>
- Sucheta, Chaturvedi, K., Sharma, N., & Yadav, S. K. (2019). Composite edible coatings from commercial pectin, corn flour and beetroot powder minimise post-harvest decay, reduces ripening and improves sensory liking of tomatoes. *International Journal of Biological Macromolecules*, 133, 284-293. <https://doi.org/10.1016/j.ijbiomac.2019.04.132>
- Trinetta, V., Morgan, M. T., Coupland, J. N., & Yucel, U. (2017). Essential oils against pathogen and spoilage microorganisms of fruit juices: Use of versatile antimicrobial delivery systems. *Journal of Food Science*, 82(2), 471-476. <https://doi.org/10.1111/1750-3841.13614>
- Yousuf, B., Wu, S., & Siddiqui, M. W. (2021). Incorporating essential oils or compounds derived thereof into edible coatings: Effect on quality and shelf life of fresh/fresh-cut produce. *Trends in Food Science and Technology*, 108, 245-257. <https://doi.org/10.1016/j.tifs.2021.01.016>

Review Article

Current Trend and Future Perspective of Shrimp Aquaculture in Malaysia: A Review

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ABSTRACT

The requirement for high-quality shellfish aquaculture production, especially shrimp, is increasingly in demand. Currently, shrimp aquaculture has become one of Malaysia's food security measures, which is consistent with the Sustainable Development Goals (SDGs) adopted by the United Nations' 2030 vision for food security and zero hunger. The outbreaks of diseases such as Acute hepatopancreatic necrosis disease (AHPND), Early mortality syndrome (EMS), White spot syndrome virus (WSSV), and infestation of virulence vibriosis bacterial, *V. parahaemolyticus* and *V. harveyi* contributed a significant loss in the shrimp aquaculture production all around the world, as well as shrimp aquaculture production in Malaysia.

Due to its major importance, the objectives of this review paper is to introduce the shrimp and prawn cultures in Malaysia emphasized on the current technology and research developed in shrimp aquaculture in Malaysia including the application of biofloc technology, the therapeutics treatment application in shrimp culture, monosex culture of all males using neo-female technology, the current trend of the shrimp production in Malaysia and on the future perspective of shrimp aquaculture in Malaysia. This review paper will generate more information, knowledge, application technology

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and beneficial findings on the shrimp aquaculture, especially for farmers and aquaculturists, in order to generate a high-quality seafood production of shrimp in the future and also to achieve the SDGs goals as well.

Keywords: Aquaculture, current technology, current trends, future perspective, Malaysia, shrimp

INTRODUCTION

Global food production systems have undergone substantial transformation, where aquaculture sectors have emerged as a leading contributor to the supply of proteins from aquaculture products. Its rapid expansion has played a significant role in strengthening the seafood security, supporting economic development, and providing essential nutrition, particularly in developing countries (Food Agricultural Organisation [FAO], 2018; Naylor et al., 2000). Within this growing sector, shrimp aquaculture has become one of the most economically valuable industries, supported by strong export demand, technological progress, and increasingly intensive production practices (FAO, 2018). By the year 2050, aquaculture production is expected to produce up to 129,000 Kt to support the world population's demand (Boyd et al., 2022). As a result, shrimp farming now represents a major source of seafood production and revenue generation, especially across Southeast Asian countries. In Malaysia, shrimp aquaculture is an essential component of the aquaculture industry and contributes directly to the national food security initiatives. The expansion of farming activities involving key commercial species such as Tiger shrimp, *Penaeus monodon* and Whiteleg shrimp, *Litopenaeus vannamei*, has been driven by their high profitability and suitability for intensive aquafarming systems (Liao & Chien, 2011). However, maintaining sustainable shrimp aquaculture production remains a challenging issue due to the increasing environmental pressures, widespread disease, and limitations in biosecurity implementation.

The occurrence of infectious diseases continues to be a major obstacle in shrimp aquaculture worldwide. Diseases and pathogens outbreaks such as White Spot Syndrome Virus (WSSV) and bacteria responsible for Acute Hepatopancreatic Necrosis Disease (AHPND) have caused widespread mass mortality and significant economic damage in leading shrimp-producing regions, including Malaysia (De Schryver et al., 2014; Lightner, 2011; Tran et al., 2013). The rapid spread and recurring nature of these diseases demonstrate the susceptibility of high-density farming systems and highlight the importance of strengthening health management frameworks (Flegel, 2012; Stentiford et al., 2012). In response, the industry has adopted various innovations and technologies aimed at improving production efficiency and environmental sustainability. Biofloc technology, for example, has gained importance as an eco-friendly technology and innovative aquaculture technology system from the application of natural microbial biotechnology that has proven to enhance

the good water quality, facilitate nutrient recycling, prevent pathogenic and disease outbreaks, as well as help minimise the reliance on conventional feed inputs (Manan et al, 2017). Furthermore, advancements such as the use of probiotics, refined pond management strategies, and selective breeding techniques have contributed to improving resilience against diseases and better overall aquaculture productivity (De Schryver et al., 2014).

Despite these developments, several issues persist within the Malaysian shrimp aquaculture sector, including disease control, environmental impacts, and consistency in the shrimp aquaculture production performance. Therefore, it is necessary to critically examine current industry practices, recent technological and innovation progress, and future development and trends pathways in the shrimp aquaculture field. This review aims to provide a comprehensive assessment of shrimp aquaculture in Malaysia, focusing on present trends, technological innovations, and future directions to support the development of a sustainable and resilient industry aligned with global food security goals.

SHRIMP AND PRAWN CULTURED IN MALAYSIA

In the 1990s, the main production of marine shrimp in Malaysia was dominated by *Penaeus monodon* (Giant Tiger Shrimp), which is popular among Malaysian farmers. Throughout the years, with the increase in stocking density, farmers have witnessed frequent shrimp diseases in the farm. In 1999, the occurrence of white spot syndrome virus (WSSV) badly hit *P. monodon* in Peninsular Malaysia and caused major loss in the shrimp production during that time. Due to this, farmers shifted their interest into culturing specific pathogen-free (SPF) *Penaeus vannamei* (Whiteleg shrimp). Over 20 years later, rapid production of *P. vannamei* could be seen, which has contributed 72% of the production, followed by 21% of *P. monodon* and 1% of *Penaeus merguensis* (Chowdhury et al., 2013). The morphological characteristics of shrimp and prawn cultured in Malaysia are as described in Table 1.

Penaeus monodon

Penaeus monodon is one of the largest penaeid shrimp in the world and has dominated the shrimp aquaculture production in Malaysia in the 1990s. It is very popular among Malaysian farmers due to its high market value. In the wild, adult *P. monodon* are found only in tropical marine areas. Meanwhile, larvae, juveniles, adolescents, and sub-adults usually will grow in the estuaries, coastal lagoons and mangrove areas. It is also a nocturnal species, which means they prefer to burrow into the bottom substratum during the day and emerge at night in search of food as a benthic feeders. In nature, *P. monodon* is likely more of a predator rather than an omnivorous scavenger or detritus feeder than other penaeid shrimp (FAO, 2009). The adults of *P. monodon* are often found at 20-50-meter depth in offshore water over muddy sand or sandy bottoms.

Table 1

Morphological characteristics of shrimp and prawn cultured in Malaysia (Fisheries Research Institute Malaysia [FRIM], 2021)

Common Name	Scientific Name	Morphological Characteristic
Black Tiger shrimp	<i>Penaeus monodon</i>	Body green-grey or dark greenish blue. Becoming reddish brown in large adults; the carapace is covered with mud-yellow transverse bands. Carapace with grooves and crest distinct; rostrum with 6-8 teeth.
Whiteleg shrimp	<i>Penaeus vannamei</i>	Body colour translucent white, but can change depending on feed, substrate and water turbidity. Body laterally compressed, elongated decapods, well-developed abdomen, adapted for swimming, rostrum moderately long, 7-10 dorsal teeth.
Banana shrimp	<i>Penaeus merguensis</i>	It has 6-9 dorsal teeth. Rostrum with a high blade and teeth above and below. Hepatic crest absent, and gastro-orbital ridge absent or very feebly defined.
Giant Freshwater prawn	<i>Macrobrachium rosenbergii</i>	It has a very long rostrum, 11-14 dorsal teeth; the male is larger than the female of the same age. An adult male has very long second chelipeds.

The mating and spawning usually occur at night shortly after moulting, while the cuticle is still soft. Subsequently, sperm kept in a spermatophore (sac) will be inserted inside the closed lycum of the female. The fertilisation occurs externally with the female suddenly extruding sperm from the lycum, and eggs are then laid in offshore waters. The eggs usually will hatch out after 12-15 hours of fertilisation (FAO, 2009). Due to persistent demand from the global market, *P. monodon* has been commercially cultured by the farmers. The increasing density and high production of *P. monodon* have led to disease outbreaks in the commercial farms. The white spot syndrome (WSS) was first observed in Peninsular Malaysia in 1994, affecting more than 80% of the shrimp farms, especially during the rainy season. The shrimp will die within 3 to 7 days after the appearance of white spots on the cuticle (Wang et al., 2000).

Penaeus vannamei

The devastating effect of white spot syndrome virus (WSSV) on the production of *Penaeus monodon* has encouraged Malaysian farmers to shift their shrimp production to the *P. vannamei*. This species has shown a faster growth rate, higher survival rate, high tolerance to ammonia and nitrate, and a wide range of adaptation to different salinities. Due to this, specific pathogen-free (SPF) *P. vannamei* was then introduced in Malaysia in 1999 (Kua et al., 2018).

Till then, production of *P. vannamei* in Malaysia has increased successfully, with the total production in 2010 reaching 69,084 tonnes, which is 50 times higher than *P. monodon* (Kua et al., 2018).

However, *P. vannamei* is non-indigenous to Malaysia, and concerns about disease outbreaks listed by the World Organisation for Animal Health (OIE), for example, Taura syndrome virus (TSV), infectious myonecrosis (IMNV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) may pose a greater risk to aquaculture in Malaysia. In 2006, Indonesia reported mass mortality of *P. vannamei* caused by IMNV. Three years later, IMNV and TSV were reported to have caused mass mortality in cultivated *P. vannamei* in Indonesia. While in Malaysia, it was reported that there were multiple bacterial infections, which included *V. parahaemolyticus* in the farms (Kua et al., 2018).

As the disease in the cultivation of shrimp farming continues to affect the shrimp production in Malaysia, attempts are required to implement biosecurity strategies in the culture system. The status of SPF *P. vannamei* only refers to its sanitary status and is not hereditary. The SPF *P. vannamei* means that the shrimp is free from certain pathogens, but is not free from all pathogens. Therefore, if the SPF shrimp is exposed to a pathogen, the SPF status is no longer valid. Thus, the implementation of biosecurity in the shrimp cultivation needs to be regularly audited to ensure a low risk of disease outbreaks in the future.

Penaeus merguensis

The *Penaeus merguensis* is one of the commercial shrimp species in the region of Indo-Pacific, including Australia, New Guinea, Indonesia, Malaysia, the Philippines, India, and Pakistan (Manan et al., 2012). Unfortunately, this species has been overlooked by farmers due to its slow growth rate compared to *P. monodon*. According to Hoang et al. (2002), the distribution of *P. merguensis* in the subtropical south-east Queensland implies its ability to withstand cold water temperature. The *P. merguensis* has been observed to withstand temperatures not lower than 9 °C and temperatures above 18 °C, which is found to consume a wide range of food, for example, crustacean, vegetable matter, phytoplankton, squids and molluscs.

However, the selection of natural food is still necessary to enhance the reproductive performance of *P. merguensis*. The production of high-quality larvae and post-larvae of *P. merguensis* is highly dependent on the broodstock condition. Memon et al. (2012) found that *P. merguensis* fed with fresh squid has a stronger influence on increasing spermatophore quality. It was observed that *P. merguensis* produces fully matured sperms at the 6th week after being fed with fresh squid. Attempts have been made by Manan et al. (2012) to induce triploidy in *P. merguensis*. The result showed that a temperature of 15 °C for durations of 10, 15, and 20 minutes is the optimum parameter to induce triploid in *P. merguensis* by using cold shock treatment.

Macrobrachium rosenbergii

In Malaysia, *Macrobrachium rosenbergii* is locally known as “udang galah”. It is popular among Malaysian farmers and is now being cultured on a large scale. The *M. rosenbergii* is a native species in Southeast Asia and is the most important aquaculture species. It is also known as the largest freshwater prawn species. The adult male of this freshwater prawn, *M. rosenbergii*, has been reported with a total body length of 35cm, and the adult female can reach about 29cm (FAO, 2002). The larvae of *M. rosenbergii* need brackish water to survive during early development. Therefore, during spawning season, fully mature females of *M. rosenbergii* will migrate from freshwater to the estuarine. The free-swimming larvae hatched from the eggs will stay attached to the female abdomen. After three to six weeks, the larvae will metamorphose into post larvae and migrate upstream towards freshwater (FAO, 2002). However, without exception, *M. rosenbergii* is also susceptible to disease caused by the parasites, bacteria and also viruses (Table 2).

TECHNOLOGY AND RESEARCH DEVELOPED FOR SHRIMP AQUACULTURE IN MALAYSIA

Biofloc Technology for Shrimp Cultures

Shrimp aquaculture is an essential sector of the Malaysian agriculture industry, contributing significantly to socio-economic development. Among cultured species, the Whiteleg shrimp (*Penaeus vannamei*) is increasingly dominant, representing a key brackish water species and the main contributor to national shrimp production (Ghee-Thean et al., 2016). Due to its growing importance, bio-secured farm designs combined with biofloc technology are well-suited for shrimp aquaculture (Taw et al., 2011). Biosecurity measures include 250-micron mesh screening for water, crab fencing, bird deterrents, water ageing, and controlled movement of farm workers, equipment, and visitors (Taw et al., 2011).

Table 2
List of common diseases in *M. rosenbergii* (FAO, 2022)

Type of Infection	Common disease
Parasites	Protozoan infestation E.g.: <i>Zoothamnium</i> sp., <i>Vorticella</i> sp., and <i>Epistylis</i> sp.
Bacteria	E.g.: <i>Vibriosis</i>
Virus	E.g.: <i>Macrobrachium</i> hepatopancreatic parvo-like virus (MHPV), <i>Macrobrachium</i> muscle virus (MMV), infection hypodermal & hematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), <i>Macrobrachium rosenbergii</i> nodavirus (MrNV), extra small virus-like particle (XSV).

Taw et al. (2011) reported significant production improvements compared to previous years, with shrimp reaching 18.8 g in 90 days at a stocking density of 130 PL/m² using biofloc technology. Taw (2017) further noted the successful implementation of a bio-secured modular recirculating aquaculture system (RAS) combined with biofloc technology at Blue Archipelago Farm since October 2011, with no reported cases of EMS or AHPND. This system was designed to treat incoming and wastewater, enhancing biosecurity and disease prevention.

Meanwhile, the Fisheries Research Institute in Gelang Patah, Johor, Malaysia, is developing a new breeding technology to enhance prawn production. This three-year pilot project, involving three prawn breeders, will be tested in Johor and Pahang to support income generation for local fishermen and farmers. Biofloc technology, comprising diverse microbial communities, is recognised as a green technology that reduces environmental impact and enhances aquaculture productivity (Kasan et al., 2017). Biofloc aggregates also contain phytoplankton, zooplankton, protozoa, nematodes, and algae, serving as an additional protein-rich feed source for shrimp (Manan et al., 2017).

Biofloc-associated bacteria such as *Halomonas venusta*, *Halomonas aquamarina*, *Bacillus infantis*, *Bacillus cereus*, *Nitratireductor aquimarinus*, and *Pseudoalteromonas* spp. are known bioflocculant producers that support early biofloc formation and improve water quality for sustainable aquaculture (Kasan et al., 2017). Another study by Manan et al. (2022) identified 43 bacterial species in *P. vannamei* biofloc culture ponds, including *Exiguobacterium* spp., *Bacillus* spp., *Vibrio* spp., *Acinetobacter junii*, *Cobetia marina*, *Rheinheimera aquimaris*, and *Pseudoalteromonas* spp., primarily isolated using HPC agar. The bacteria *Exiguobacterium aestuarii* and *Exiguobacterium profundum* were observed to be dominant in the shrimp biofloc pond sample, where these organisms are capable of producing the extracellular enzyme activity to break down the chemical structures, thus benefiting in eliminating the organic compounds from the aquaculture waste.

According to Taw (2013), the application of biofloc technology for biosecurity could enhance the sustainable aquaculture production by reducing the outbreak of disease outbreak problems and the cost of energy in the shrimp farm operation in Arca Biru Kedah shrimp Farm, Blue Archipelago, Malaysia. The application of biofloc technology also really contributed to natural bioremediation in the zero or minimal water exchange system, improving the shrimp growth performance (Manan et al., 2020) and maintaining the water quality at the optimum level until the end of the culture period (Manan et al., 2017).

Therapeutic Treatment in Shrimp Aquaculture

On the other hand, a research study on a small scale was conducted in the laboratory by Ikhwanuddin et al. (2014) on the therapeutic activity effect of Indian almond, *Terminalia catappa* leaves extraction on the survival rate and growth performance of Tiger shrimp,

Penaeus monodon postlarvae. It was identified that a 3.0 mg/L concentration of *T. catappa* leaves extraction showed a positive effect on the survival rate and growth performance of *P. monodon* postlarvae. Meanwhile, Kawamura et al. (2019) identified that peppermint, *Metha piperita*, enhanced the feeding activity on giant freshwater prawn, *Macrobrachium rosenbergii*, in all stages except during larvae stage, and the garlic application also enhanced the feeding for the freshwater prawn during juveniles and postlarvae stages. Syahidah et al. (2015) found that garlic was identified as a strong food calling effect as an appetite stimulator for fish, and the bioactive compound in garlic, known as alicin, was identified to help in food ingestion and increase the feed intake in fish. On top of that, the red chilli, *Capsicum annuum*, was identified as the best feeding attractant for Whiteleg shrimp, *L. vannamei* and ginger, *Zingiber officinale* and papermint as moderate attractants for adults and juvenile shrimp of *L. vannamei* (Kawamura et al., 2019). The therapeutic effect from natural plants was reported to promote positive effects such as stress reduction, promoting growth, appetite stimulator and promoting immunostimulants in aquatic animals (Citarasu, 2010). Several herbal supplements inclusion in the food conducted in 90 days trial also identified help enhance the food intake in crustacean like ginger (*Zingiber officinale*), turmeric (*Curcuma longa*) and garlic (*Allium sativum*) help enhanced the food intake in giant freshwater prawn, *M. rosenbergii* (Rebecca & Bhavan, 2014). The turmeric, *Curcuma longa*, also proven contains a good source of antioxidants and helps increase the survival rate of giant freshwater prawn, *M. rosenbergii* (Rebecca & Bhavan, 2014).

The Use of Probiotics in Shrimp Aquaculture

A study conducted by Mohamad et al. (2020) on the effects of potential probiotics of Lactic Acid Bacteria (LAB) of *Lactobacillus plantarum* and *Enterococcus faecalis* isolated from *M. rosenbergii* prawn found that these probiotics help in improving the aquaculture species growth performance, survival rate, and also help improve the immunity response towards disease infection in the aquaculture operation. Andani et al. (2012) identified that most of the probiotics are supplied as live supplements in the feed to benefit the culture organisms by inhibiting the pathogenic microbes, improving the immunity, survival and growth rate, enhancing the digestion and feed utilisation of the culture organisms, as well as helping improve the water quality in the culture system.

The application of probiotics in aquaculture helps degrade the organic matter in the pond water, stabilises the microbial community for the health ecosystem and also helps maintain the natural environments in the safety and optimum level for the shrimp culture. Currently, many shrimp farmers are applying the new technology of probiotics in their shrimp farms to improve the farm production yield with good aquaculture practices for the sustainability of seafood safety and quality. For example, the probiotics from the *Bacillus subtilis* strain can help inhibit the effect against vibriosis infection in Tiger shrimp, *Penaeus monodon* (Vaseeharan & Ramasamy, 2003).

Monosex Culture Technology

The production using monosex culture also comes into focus as the technique helps increase the aquaculture production (Manan & Ikhwanuddin, 2021). The application of triploidy to the penaeid shrimp in the laboratory scales seems to help increase the shrimp size, faster the shrimp growth rate and also successfully induce monosex culture as well as other techniques of polyploidy, gynogenesis and hormone treatments (Manan & Ikhwanuddin, 2021). Another study conducted by Ikhwanuddin et al. (2019) identified 100% feminisation of *Penaeus merguensis* postlarvae using 17β -estradiol, an estrogen hormone, with a concentration of 1600 mg/kg.

The monosex culture of all females is economically beneficial and helps increase yield production due to its faster growth rate compared to male culture or mixed cultures (Ikhwanuddin et al., 2019). Another technique using gynogenesis manipulation also could be applied to generate a monosex culture of all-female type offspring with diploid gynogens of fish and crustacean produces (Manan et al., 2020). The monosex culture of all female type offspring from the gynogenesis technique, it could produce female gynogens with better size than males and subsequently can help increase the market size, yield and profitability (Manan et al., 2020).

Micro Bubble Aeration Technology

The application of a micro bubble aeration system was applied in the nursery and pond of shrimp culture in Malaysia in order to help reduce the cost and environmental impact of the shrimp farming. The application of this newly efficient technology is to reduce the amount of water required for shrimp aquaculture by improving the water cleanliness and reducing the effluent and costing for effluent treatment. *M. rosenbergii*, or locally known as the Giant freshwater prawn, is one of the essential commercial aquaculture species that is being given a top priority by the Department of Fisheries Malaysia (DOF) as a seafood product for consumer consumption and also for exportation (Banu, 2014).

Giant freshwater prawn was commonly found in the river and estuaries of tropical and subtropical countries where this species was identified as a significantly important culture species in many countries such as China, India, Bangladesh, Thailand, Vietnam, Malaysia and Brazil (Tan et al., 2020). Asaduzzaman et al. (2006) identified that the culture of all male prawn, *M. rosenbergii*, resulted in higher production than the mixed culture. Banu (2014) conducted research on male Giant freshwater prawn, identifying that male juveniles of *M. rosenbergii* were homogenous after the cold shock treatments and, on the other hand, were heterogeneous after being treated with 17α methyl testosterone hormone.

Neo-female Technology Application for the Production of all Male *M. rosenbergii* Prawn

Males of *M. rosenbergii* grew faster and reached a larger size during harvest compared to the females of the same species. A culture of all-male prawns can increase both production and profit because male prawns can exhibit a faster growth rate compared to female prawn from similar age (Tan et al., 2020). Therefore, a monosex culture of all male prawns would be advantageous to the economic increments. Sagi and Cohen (1990) found out that in crayfish, lobster, spiny lobsters, shrimp and prawn have a WZ-ZZ scheme, where male identified as the homogametic sex. It was identified that the androgenic gland (AG) in crustacean endocrine produced insulin-like androgenic gland (IAGO) hormone for sex differentiation, where without this AG or a deficiency of IAG will result in feminisation (Levy & Sagi, 2020).

Removal of the AG through the AG ablation or microsurgical intervention from the immature male will result in sex reversal into neo-females, which are capable of mating with normal males to produce all-male offspring (Sagi & Aflalo, 2005). Due to its importance, GK Aqua Sdn. Bhd. located in Peninsular Malaysia, was established in 2016 and currently applies the neo-female technology to produce all male *M. rosenbergii*. GK Aqua imported the neo females post larvae from the hatchery of China and Vietnam, where this PL was produced through the gene silencing technology and a complete sex reversal from male into functional neo-females. This neo female broodstock could produce up to 100% male offspring due to the chromosome pairing during mating with the normal male prawn.

On the other hand, GK Aqua also try to produce the neo-female by injecting RNAi silencing chemical, also known as insulin-like androgenic gland hormone, into the normal male prawn, that prevented it from being male and then sex reversal into a neo-female. The use of the RNAi injected into the prawn seems to be safe due to its temporary nature, where the exogenous dsRNA was fully cleared from prawn tissue within 7 days (Lezer et al., 2015). This technique is not a genetically modified product, as there are no hormones or chemicals used. About 1.2 metric tons of all male prawns can be produced every six months with the good survival rate between 60% to 70% of survival (Habib, 2017).

PRODUCTION OF SHRIMP AQUACULTURE IN MALAYSIA

Global aquaculture production continues to rise, with shrimp farming being a major contributor (Hidayati et al., 2020). Since its emergence in the 1920s, aquaculture has grown rapidly and is now a key sector for economic development and food security in Malaysia. Surrounded by rich water bodies, Malaysia hosts diverse shrimp species and ranks as the ninth-largest shrimp exporter globally. The demand for Malaysian shrimp has increased steadily since the 19th century, coinciding with the growing popularity of aquaculture.

Shrimp aquaculture plays a crucial role in Malaysia's agricultural sector by contributing to food supply, GDP growth, employment, and economic development (Ghee-Thean et al., 2016; Lee et al., 2020). There were several main commercial shrimp or prawn species that contribute as a vital economic pillar in the industry of many countries, including Malaysia, which are *Penaeus monodon*, *Penaeus vannamei* and *Macrobrachium rosenbergii* (Soo et al., 2019). Among these, *P. vannamei* is the dominant species in brackish water culture, accounting for 89% of total farmed shrimp production, followed by *P. monodon* (10%) and *M. rosenbergii* (1%) (Ghee-Thean et al., 2016; Muthukrishnan et al., 2019).

Shrimp farming in Malaysia covers an estimated 5,100 hectares (Chowdhury et al., 2013). In 2020, brackish water and freshwater aquaculture productions were 302,807 tonnes and 97,210 tonnes, respectively (Department of Fisheries Malaysia [DOF], 2020). Aquaculture in Malaysia is categorised into brackish water, freshwater, and marine culture, with brackish water aquaculture being the dominant practice (FAO, 2022; Lee et al., 2020). The main cultured species include Tiger shrimp (*Penaeus monodon*), White shrimp (*Penaeus vannamei*), and Giant freshwater prawn (*Macrobrachium rosenbergii*), primarily farmed in pond systems (DOF, 2020; Ismail & Abdullah, 2013).

Brackish water shrimp, including *P. monodon* and *P. vannamei*, contribute 49.80% to the wholesale value of aquaculture production in Malaysia (DOF, 2020). In 2017, shrimp and prawn exports, including live, fresh, or frozen forms, totalled RM1.2 billion from 51,481 tonnes of production (DOF, 2017). These exports mainly went to Singapore, Vietnam, Australia, China, Japan, Taiwan, Korea, Hong Kong, and the European Union. While Tiger shrimp production has gradually increased over the past decade, it remains lower than that of White shrimp.

The production of White shrimp and Giant freshwater prawn significantly increased between 2013 and 2014 but declined substantially in 2020 (DOF, 2011-2020) (Figure 1). This decrease was due to the global COVID-19 pandemic, which disrupted economic activities, including Malaysia's aquaculture sector, as countries imposed strict quarantine measures (Pazir et al., 2022; Waiho et al., 2020). According to a FAO (2020) report, high-value commodities like shrimp were particularly vulnerable to COVID-19, facing export limitations from flight closures and a major decline in sales to hotels and restaurants.

As shrimp is the most traded fishery commodity in Malaysia, that contribute to the 53400 tonnes with a total wholesale value production of RM1.2 million (49.77% of total aquaculture production) in 2019 (DOF, 2019), this pandemic has adversely impacted the national economy of Malaysia as shown in Figure 1. Past research analysis across 26 countries also showed that this global pandemic severely disrupted and significantly decreased business sales during the lockdown or during the Malaysian movement control order (MCO) periods (De Vito & Gomez, 2020). Among the documented affected countries of shrimp production are Bangladesh shrimp (Rahman et al., 2021) and Iran shrimp (Pazir et al., 2022), as the demand for seafood has reduced and led to the collapse of the supply chain drastically.

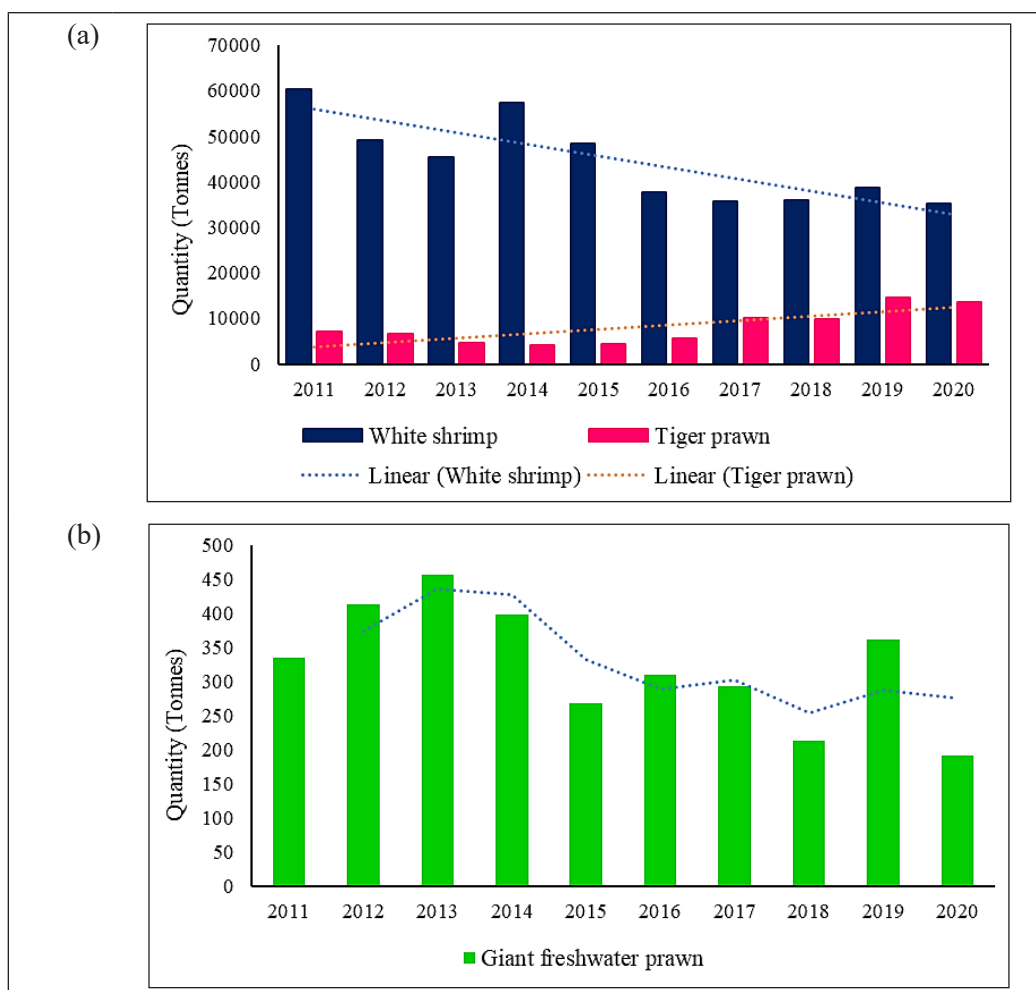


Figure 1. Aquaculture production of (a) White shrimp and Tiger prawn from brackish water culture system and (b) giant freshwater prawn from freshwater culture system from 2010 to 2020. (Department of Fisheries Malaysia, 2019)

However, during the COVID-19 pandemic lockdown, the aquaculture sector was given permission by the government to re-establish the new normal operation to ensure food security in Malaysia (Waiho et al., 2020). Although the total of white shrimp production decreased during COVID-19, some states of Malaysia showed improved culture production, such as Perlis, Perak, Selangor, Johor, Terengganu, Kelantan and Sarawak. Meanwhile, for tiger shrimp, Perlis, Kedah, Negeri Sembilan, Melaka, Johor, Pahang and Terengganu exhibited an increase in culture production. The aquaculture production of giant freshwater prawn indicated a decline for most of the states, except Kedah, which showed an increase in production (DOF, 2019; 2020) (Figure 2).

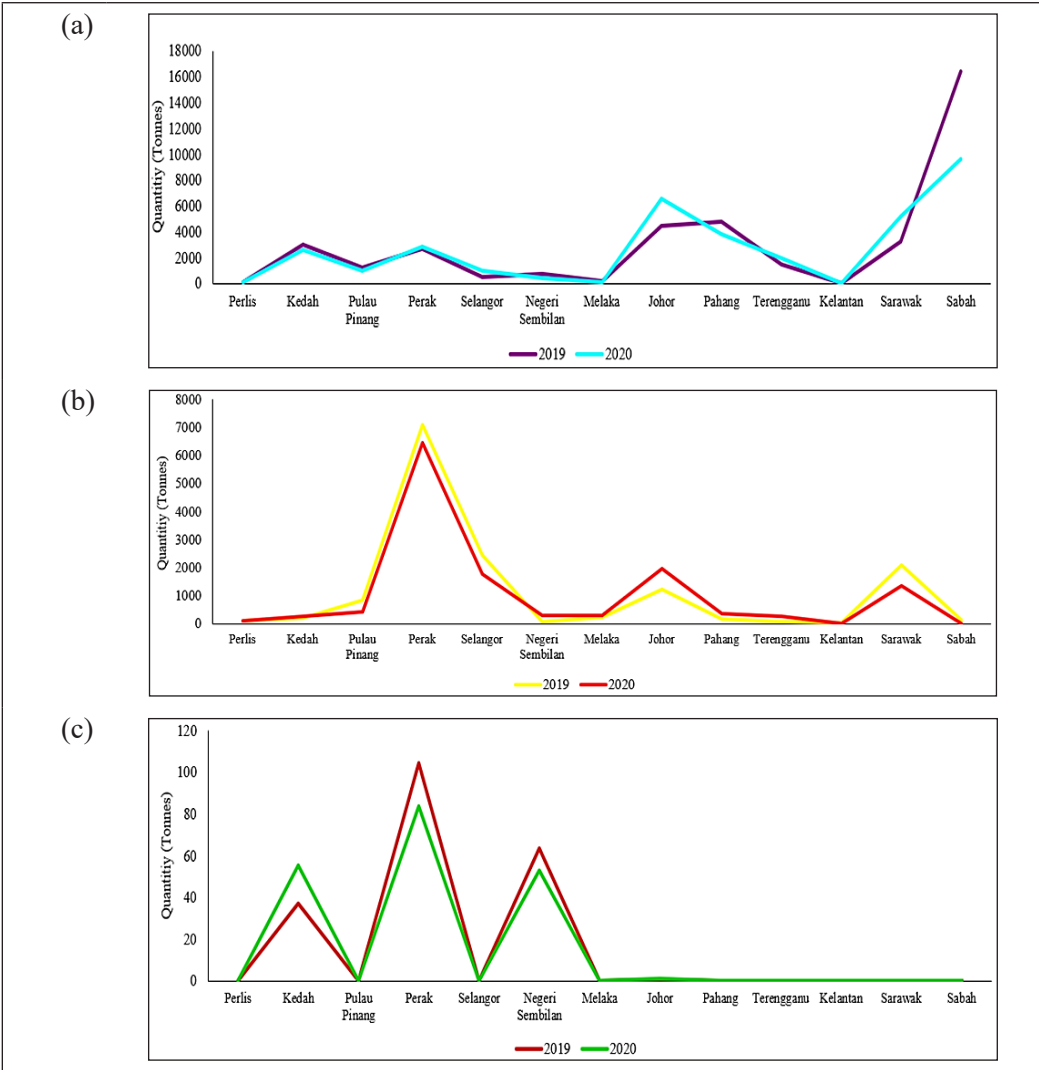


Figure 2. Aquaculture production of shrimp by state and species (a) white shrimp, (b) tiger prawn and (c) giant freshwater prawn in 2019 to 2020 (Department of Fisheries, 2019; 2020)

Five components in the marketing channel have been practised for the production of aquaculture white shrimp, which are the shrimp farmer, distributor, wholesaler, retailer and consumer (Lee et al., 2020) (Figure 3). The shrimp farmer sells the shrimp to the distributor for RM16 per kilogram, and the distributor then sells the shrimp to the wholesaler for RM18 per kilogram. While white shrimp sell for RM20 per kilogram at wholesale, they cost RM23 per kilogram at retail price. The total cost of marketing is RM7 per kilogram. Due to the fact that there is little gap between the channels, the marketing channel is highly effective (Lee et al., 2020).

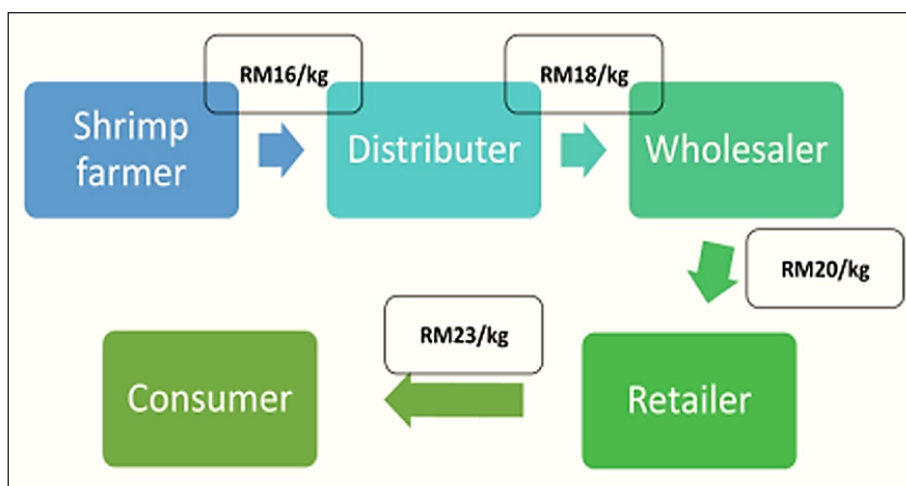


Figure 3. Five components involved in the marketing channel for the production of white shrimp in Kedah started from the shrimp farmer, until reaching the consumer (Lee et al., 2020)

Therefore, with the new strategies and implementation by the Malaysian government and other agencies in supporting the economic initiatives from production to marketing strategies, it is expected to decrease the distractions and losses caused by COVID-19 (Waiho et al., 2020).

CURRENT TREND IN SHRIMP PRODUCTION IN MALAYSIA

Status of Shrimp Production in Malaysia

Shrimp production in Malaysia has steadily increased since the 19th century, contributing to its status as the 9th largest shrimp exporter globally. Currently, 45% of Malaysia's shrimp production comes from capture fisheries, and 55% from aquaculture (Mohd-Haikal & Mohammad-Mustafizur, 2019). With a coastline of 4,809 km and a land area of 329,750 km², Malaysia has developed 5,100 hectares for shrimp farming, including both brackish water and freshwater culture (Chowdhury et al., 2014). The first marine shrimp farm was established in Johor in the 1930s and upgraded to semi-intensive culture in the 1970s (FAO, 2022).

The establishment of privately-owned shrimp hatcheries, farms, and food factories in the 1980s helped commercialise aquaculture in Malaysia. Currently, shrimp culture has experienced a 10% annual growth rate over the past five years, evolving into a profitable and sustainable industry, particularly with high-value species. In 2020, Malaysia's largest shrimp production was from White shrimp, with 35,063.90 tonnes from ponds and 84.75 tonnes from tank systems. Following disease outbreaks in 2001, production shifted from Tiger shrimp to White shrimp, which now constitutes about 67% of aquaculture shrimp

production (Chowdhury et al., 2014). In 2002, aquaculture exports, including shrimp, to Japan totalled 198,892 tonnes, valued at RM1.5 billion.

Then, there were 94 shrimp hatcheries listed in 2003, with an estimated total production of 12 billion fries per year (Mazuki & Subramanian, 2005). In 2010, the total production of marine shrimp from Malaysia reached 87,202 tonnes with a total value of 0.35 billion USD, and simultaneously with that, Malaysia successfully listed as one of the top countries in the world's shrimp production (Chowdhury et al., 2014). The main culture system practices for shrimp culture in Malaysia are ponds, concrete tanks and cages, while the largest harvest is mainly obtained from brackish water. According to the Department of Fisheries Malaysia statistics 2020, the large share of giant freshwater prawn and crayfish harvest in 2020 came from the freshwater pond culture system, which is 191.32 tonnes and 57.43 tonnes respectively, compared to freshwater tank culture, which only produced 1.37 tonnes of Giant freshwater prawn and 6.95 tonnes of crayfish.

Meanwhile, the biggest production of the brackish water species also mostly comes from the pond culture system, which is tiger shrimp, and white shrimp produced 13,524.76 tonnes and 35,063.90 tonnes, respectively, as compared to the tank culture system, which only produces white shrimp with the production of 84.75 tonnes. On the other hand, the lobster culture in brackish water mostly comes from the cage culture system, with total production in 2020 being 0.22 tonnes. The selection of ponds (earthen pond or semi-earthen pond) as an aquaculture system for shrimp culture is more popular in Malaysia, probably due to the simple design and easy to build, low construction costs, long-lasting system, larger stocking density per pond, can generate live feed which can support nutrition supply to the livestock and low maintenance costs. Furthermore, the clay soil can stabilise the bottom of the earthen pond and absorb numerous nutrients and organic matter, thus increasing the productivity of the pond (Khai et al., 2011).

Based on Annual Fisheries Statistics (DOF, 2016; 2017; 2018; 2019; 2020) (Table 3), the overall total production of shrimp was the highest in 2019, with the production of 53,619.28 tonnes valued at RM1,169,099.61 and at the lowest in 2016 with the production of 43,563.04 tonnes valued at 9,355,982.34. The production of freshwater shrimp is highest in 2016 with a total production of 309.34 tonnes valued at RM7,350.34, and at the lowest is in 2019 with a total production of 205.58 tonnes valued at RM12,757.12. Then, the production in 2020 increased by 25.05%, which is 257.07 tonnes valued at 16,265.23. Meanwhile, the production of marine shrimp started low in 2016 with a total production of 43,253.61 tonnes valued at RM43,252.61 and gradually increased in the following year and reached the highest production in 2019, which is 53,413.7 tonnes valued at RM1,257,745.91.

The marine shrimp production dropped by 8.87%, which is 48673.63 tonnes valued at RM1,169,099.61. In conclusion, the trend of freshwater shrimp production seems to drop gradually starting in 2016 until 2019 and then increases in 2020, while in contrast

with marine shrimp, which gradually increases from 2016 until 2019 and drops in 2020. A good aquaculture production strategy is determined by the proper operational management, stocking density, production targets and economic achievements planned by the breeder/pond operator. According to Chowdhury et al. (2014), the shrimp farming industry in Asian countries, including Malaysia, China, Thailand, and Indonesia, has experienced and fought the situation of “up and down” in their production due to massive viral disease attacks as well as fluctuation in the global market price. Table 3 shows the total Malaysian aquaculture shrimp production from 2016 to 2020.

The production of shrimp fries and hatchlings from government and private hatcheries from 2016 to 2019 for aquaculture purposes is shown in Table 3. It was shown that private hatchery production is greater than that of the government hatchery. The government hatchery produced 27,302,920 fries in 2017, which is the highest production compared to other years, but drastically declined to the following year of 2018 and 2019, which are 11,915,760 and 11,935,847 fries, respectively. In the meantime, the production of private hatchery was the highest in 2016 (9,111,575,183 fries), and the lowest was in 2017 (4719, 259,449 fries). Overall, the total production of fries and hatchlings from both sectors is at the highest in 2016 (9,111,575,183 fries) and at the lowest in 2017 (4719,259,229 fries). However, the number of fries produced does not seem to be well-linked to the total production of shrimp all year (Table 4), which means that the highest fry production does not guarantee large production as a result. The final result depends on the proper management skills and the quality of the shrimp (high durability).

Table 3
Total Malaysia's aquaculture production of shrimp and value from 2016 to 2020 (Adaptation from Department of Fisheries Malaysia 2016-2020)

Year	Species of Shrimp	Freshwater	Marine	Total
2016	Quantity (tonnes)	309.43	43,253.61	43,563.04
	Value (RM'000)	17,350.34	918,632	935,982.34
2017	Quantity (tonnes)	293.74	45,783.09	46,076.83
	Value (RM'000)	16,655.34	1,067,337.05	1,083,992.39
2018	Quantity (tonnes)	213.42	45,923.23	46,136.65
	Value (RM'000)	12,088.51	1,114,951.96	1,127,040
2019	Quantity (tonnes)	205.58	53413.7	53,619.28
	Value (RM'000)	12,757.12	1,257,745.91	1,270,503
2020	Quantity (tonnes)	257.07	48673.63	48,673.63
	Value (RM'000)	16,265.23	1,169,099.61	1,169,099.61

Table 4

Production of shrimp fries and hatchlings from government and private hatcheries from 2016 to 2019

Hatchery	Year			
	2016	2017	2018	2019
Government	17,835,183	27,302,920	11,915,760	11,935,847
Private	9,093,740,000	4,691,956,529	6,474,892,952	5,200,107,156
Total production	9,111,575,183	4,719,259,449	6,486,808,712	5,212,043,003

Note. Quantity in pieces

Production risk analysis indicates that the lack of farmers' knowledge and experience related to shrimp aquaculture management, operational skill, marketing, and accounting has a negative impact on technical inefficiency and cost inefficiency (Thean, 2014). Therefore, the Malaysian government needs to take the initiative by holding workshops and training related to aquaculture operation and marketing strategies, as well as motivational seminars to increase farmers' knowledge related to proper and efficient shrimp culture and to give mental encouragement in increasing their efficiency in managing their farming projects while obtaining higher production. Another common problem encountered by the shrimp aquaculture industry is disease outbreaks, world market price, quality of broodstock, feed availability and quality, banned drugs and antibiotics used, environmental impact and control, world trade obstacles, as well as market cooperation and public associations management, which need to be faced and resolved precisely (Vergel, 2017).

Human Resources and Economic Development Through Aquaculture and Shrimp Farming

Aquaculture generated employment for 20,976 people in 2004, and via six different culture system practices, with the highest total employment in the freshwater pond and concrete culture system, with a percentage of 70% (Figure 4). The number of aquaculturists increased from 21,100 individuals in 2005 to 21,790 individuals in 2015 but slightly decreased to 20,262 individuals in 2020 (DOF, 2020; Mazuki & Subramanian, 2005). In some areas, especially in small-scale cultural practices, the aquaculture farms are worked by family members and close relatives. The total number of aquaculture workers is mostly contributed by men, which is 90% and only 10% by women (FAO, 2022).

Lack of knowledge in aquaculture practices at the beginning of the intensive culture period in the 19th century has led to huge damage due to poor management and disease outbreaks (Shariff & Subasinghe, 1993). In view of such encounters, the availability of qualified human resources in relevant subjects and disciplines is required for sustainable development and management in the aquaculture industry.

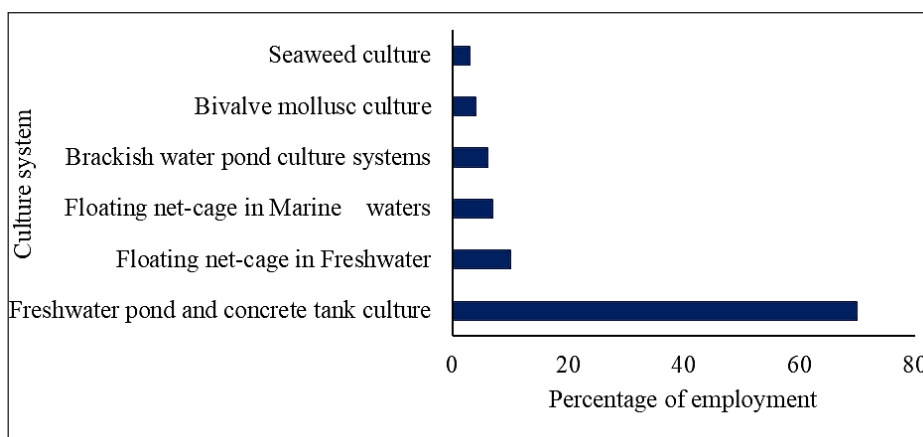


Figure 4. Percentage of employment in the Malaysian aquaculture industry in 2004 based on culture system practices (modified from FAO, 2022)

Due to this fact, in the last few years, the participation of skilled and qualified fish farmers as well as the participation of professionals from local and foreign higher institutions has brought the aquaculture industry, including shrimp farming, towards success with huge profit and sustainable outcome.

In the last two years (2019-2020), the employment in aquaculture farms and labour for shrimp marketing access in seafood industries was also disrupted by the lockdown during the pandemic, COVID-19 and served badly to the small-scale aquaculture entrepreneurs in the most developing countries, hence cutting off their incomes and also causing unemployment (Reardon et al., 2020). Nowadays, after the pandemic, the shrimp farming entrepreneurs are struggling to rebuild this industry and return to the situation it was before the pandemic.

Demand and Market Trend of Shrimp

Market Research Report (2022) states that the market size of global shrimp was estimated to expand from \$33.81 billion in 2021 to \$53.63 billion in 2028 at a Compound annual growth rate (CAGR) of 6.81% in the prediction period of 2021 to 2028. The CAGR in terms of shrimp production refers to the average yearly growth rate of a shrimp investment in a period of more than one year, and it symbolises the most precise methods to analyse and define profits for a specific property, investment matters and other matters that can develop a pattern in the revenue. The COVID-19 pandemic has caused a disturbance in the international aquaculture industry, including shrimp farming. The pandemic harshly interrupted the farming and marketing activities of seafood production.

A series of lockdowns due to the COVID-19 pandemic in most countries worldwide has contributed to the health and economic crisis that has also shaken food and nutrition safety (Sumner et al., 2020). The shrimp industry also negatively impacts its market demand and supply chain during the pandemic. The growth rate of the shrimp global market declined to 14.46% if compared to the average growth from 2017 to 2019 before the pandemic outbreak. The low market demand during the lockdown was resulting dumping of seafood supply because it could not be marketed during the lockdown, resulting in a decrease in the market trend. Nowadays, the shrimp industry is returning to the track step by step as the market demand and trend have gradually increased after the struggle against the after-effects of the pandemic.

FUTURE PERSPECTIVE IN SHRIMP AQUACULTURE IN MALAYSIA

The aquaculture of shrimp is one of Malaysia's food security measures and is consistent with the Sustainable Development Goals (SDGs) adopted by the United Nations' 2030 vision for food security and zero hunger. Strategic planning for aquaculture management to generate a large amount of shrimp with excellent quality, low mortality, and high market demand. Aquaculture products are among the essential sources of protein in the world. Implementing technology to establish a high-tech system in aquaculture management is an excellent starting point for achieving more profitability. SD-AQEP, a system dynamics model of the eco-aquaculture system developed by the local iSHARP aquaculture sector, increased the system's productivity by improving feedstock, water utilisation, and wastewater management (Isa et al., 2021).

Good aquaculture practice (GAP) is required to manage broodstock, ponds, and water quality (Samah, 2020). Even though insufficient compliance with requirements for outstanding biosecurity and effective aquaculture operations in shrimp farms and hatcheries has been identified as a significant factor in the spread of disease from one farm to another, there is still a substantial amount of work to be done. Farmers have essential responsibilities as biosecurity monitors in shrimp farms (Padilah, 2022), such as producing healthy shrimp seedlings, checking the water quality of ponds and water resources regularly, and sterilising spent nets and trawlers before reusing them in other ponds.

Acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome, is an emerging shrimp illness that has reduced worldwide shrimp (*Litopenaeus vannamei*) output by more than 60%, with annual global economic losses of up to \$1 billion predicted (Yan et al., 2019). This AHPND was caused by a virulent strain of bacteria, including *V. parahaemolyticus*, *V. punensis*, *V. harveyi*, *V. owensii*, *V. campbelli*, and *Shewanella* sp., carrying the pVA1 plasmid (63-70 kb) expressing the binary PirAVP and PirBVP toxins. Chemicals and antibiotics were widely employed in the battle against AHPND and must be regulated to prevent the creation of antibiotic-resistant bacteria, which

may pose global health risks. (Kumar, 2021). Earlier research identified tetracyclines, sulfamides, and quinolones as Malaysian aquaculture's three most prevalent antibiotics. It was also determined that the relative abundance of resistance genes declined from *sul2* > *tet(M)* > *sul1* > *sul3* and that there is no association between antibiotic residue and resistance genes (Thiang, 2021).

Future research endeavours in increased monitoring, detection, and prevention are necessary to maintain shrimp aquaculture development in Malaysia as a step toward increasing biosecurity in the country's aquaculture industry (Chiew, 2019). Marketing and sales are vital for preserving the shrimp supply to consumers and maximising profit margins. The regulation of white leg shrimp production involves two main expenditures: feed and fingerling costs, which account for about 48% of total operating costs, and 17% of overall operating costs. The nutrient-rich diet is also expensive, including shrimp's nutritional needs. It contains fish, rice bran, soy, vitamins, and minerals. However, giving a low-cost food deficient in vital nutrients causes the shrimp production cycle to expand slowly.

CONCLUSION

In conclusion, shrimp aquaculture in Malaysia plays a vital role in supporting food security and aligning with global sustainability goals, but it continues to face significant challenges from disease outbreaks that reduce productivity and profitability. Advances such as biofloc technology, improved therapeutics, and monosex culture systems demonstrate promising progress toward enhancing production efficiency. However, sustaining long-term growth requires more resilient and health-focused farming strategies. Greater emphasis should be placed on natural and sustainable approaches to disease prevention and growth enhancement.

Hypothetically, as a recommendation, future research should be conducted on the inclusion of herbal additives in shrimp feed diets, as these may improve shrimp health, strengthen immune responses, and enhance growth performance while reducing dependence on chemical treatments.

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REFERENCES

- Andani, H. R. R., Tukmechi, A., Meshkini, S., & Sheikhzadeh, N. (2012). Antagonistic activity of two potential probiotic bacteria from fish intestines and investigation of their effects on growth performance and immune response in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Ichthyology*, 28(5), 728-734. <https://doi.org/10.1111/j.1439-0426.2012.01974.x>
- Asaduzzaman, M., Wahab, M. A., Yi, Y., Diana, J. S., & Lin, C. K. (2006). Bangladesh prawn-farming survey reports industry evaluation. *Global Aquaculture Advocate*, 9(6), 40-42. <https://aquafishcrsp.oregonstate.edu/sites/aquafishcrsp.oregonstate.edu/files/nop06-a14.pdf>
- Banu, R. (2014). *Influence of heterogeneous individual growth on male freshwater prawn Macrobrachium rosenbergii De Man for sustainable monosex culture* [Doctoral dissertation, Universiti Putra Malaysia]. Institutional Repository. <http://psasir.upm.edu.my/id/eprint/70339/1/FP%202014%2071%20-%20IR.pdf>
- Boyd, C. E., McNevin, A. A., & Davis, R. P. (2022). The contribution of fisheries and aquaculture to the global protein supply. *Food Security*, 14, 805-827. <https://doi.org/10.1007/s12571-021-01246-9>
- Chiew, I., Salter, A. M., & Lim, Y. S. (2019). The significance of major viral and bacterial diseases in Malaysian aquaculture industry. *Pertanika Journal of Tropical Agricultural Science*, 42(3), 1023-1047.
- Chowdhury, M. A., Talib, A., & Yahya, K. (2013). *A review on marine shrimp aquaculture production trend and sustainability in Malaysia and the world perspective* [Conference presentation].
- Chowdhury, M. A., Talib, A., & Yahya, K. (2014). A review on marine shrimp aquaculture production trend and sustainability in Malaysia and the world perspective. *Proceedings of the Conference: International Fisheries Symposium*, Can Tho University, Can Tho, Vietnam.
- Citarasu, T. (2010). Herbal biomedicines: A new opportunity for aquaculture industry. *Aquaculture International*, 18, 403-414. <https://doi.org/10.1007/s10499-009-9253-7>
- Department of Fisheries Malaysia. (2011). *Annual Fisheries Statistics 2011*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2012). *Annual Fisheries Statistics 2012*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2013). *Annual Fisheries Statistics 2013*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2014). *Annual Fisheries Statistics 2014*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2015). *Annual Fisheries Statistics 2015*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2016). *Annual Fisheries Statistics 2016*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2017). *Annual Fisheries Statistics 2017*. Ministry of Agriculture and Agro-Based Industry Malaysia.

- Department of Fisheries Malaysia. (2018). *Annual Fisheries Statistics 2018*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2019). *Annual Fisheries Statistics 2018*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2020). *Annual Fisheries Statistics 2018*. Ministry of Agriculture and Agro-Based Industry Malaysia.
- Department of Fisheries Malaysia. (2020). *Annual fisheries statistics: Number of aquaculturists* [Perangkaan perikanan tahunan: Bilangan penternak akuakultur]. (Vol. 1, pp. 1-84). Ministry of Agriculture and Agro-Based Industry Malaysia.
- De Schryver, P., Defoirdt, T., & Sorgeloos, P. (2014). Early mortality syndrome outbreaks: A microbial management issue in shrimp farming? *PLoS Pathogens*, 10(4), Article e1003919. <https://doi.org/10.1371/journal.ppat.1003919>
- De Vito, A., & Gómez, J. P. (2020). Estimating the COVID-19 cash crunch: Global evidence and policy. *Journal of Accounting and Public Policy*, 39(2), Article 106741. <https://doi.org/10.1016/j.jaccpubpol.2020.106741>
- Food and Agriculture Organisation. (2002). *Farming freshwater prawns: A manual for the culture of the giant river prawn (Macrobrachium rosenbergii)*. Food and Agriculture Organisation of the United Nations. <https://www.fao.org/3/y4100e/y4100e00.htm#TOC>
- Food and Agriculture Organisation. (2009). *Penaeus monodon*. In *Cultured aquatic species fact sheets*. Food and Agriculture Organisation of the United Nations. http://www.fao.org/fishery/docs/CDrom/aquaculture/I1129m/file/en/en_gianttigerprawn.htm
- Food and Agriculture Organisation. (2018). *The state of world fisheries and aquaculture 2018*. Food and Agriculture Organisation of the United Nations. <https://www.fao.org/3/i9540en/I9540EN.pdf>
- Food and Agriculture Organisation. (2020). *The impact of COVID-19 on fisheries and aquaculture: A global assessment from the perspective of regional fishery bodies*. Food and Agriculture Organisation of the United Nations. <http://www.fao.org/3/ca9279en/CA9279EN.pdf>
- Food and Agriculture Organisation. (2022). *Macrobrachium rosenbergii*. In *Cultured Aquatic Species Information Programme* (Text by New, M. B.). Fisheries and Aquaculture Division. Food and Agriculture Organisation of the United Nations. https://www.fao.org/fishery/en/culturedspecies/macrobrachium_rosenbergii/en
- Fisheries Research Institute Malaysia. (2021). *FRI database system*. https://fri.dof.gov.my/fri.php/database_stores/store_view_page/85/1
- Flegel, T. W. (2012). Historic emergence, impact and current status of shrimp pathogens in Asia. *Journal of Invertebrate Pathology*, 110(2), 166-173. <https://doi.org/10.1016/j.jip.2012.03.004>
- Ghee-Thean, L., Islam, G. M. N., & Ismail, M. M. (2016). Malaysian white shrimp (*P. vannamei*) aquaculture: An application of stochastic frontier analysis on technical efficiency. *International Food Research Journal*, 23(2), 638-645.
- Hidayati, N. V., Prudent, P., Asia, L., Vassalo, L., Torre, F., Widowati, I., Sabdono, A., Syakti, A. D., & Doumenq, P. (2020). Assessment of the ecological and human health risks from metals in shrimp aquaculture

- environments in Central Java, Indonesia. *Environmental Science and Pollution Research*, 27(33), 41668-41687. <https://doi.org/10.1007/s11356-020-09967-8>
- Hoang, T., Lee, S. Y., Keenan, C. P., & Marsden, G. E. (2002). Effect of temperature on spawning of *Penaeus merguensis*. *Journal of Thermal Biology*, 27(6), 433-437. [https://doi.org/10.1016/S0306-4565\(02\)00013-X](https://doi.org/10.1016/S0306-4565(02)00013-X)
- Ikhwanuddin, M., Moh, J. H. Z., Manan, H., Noor-Hidayati, A. B., Aina-Lyana, M. A., & Nor Juneta, A. S. (2014). Effect of Indian almond, *Terminalia catappa* leaves water extract on the survival rate and growth performance of black tiger shrimp, *Penaeus monodon* post larvae. *AACL Bioflux*, 7(2), 85-93. <https://bioflux.com.ro/docs/2014.85-93.pdf>
- Ikhwanuddin, M., Bahar, H., Ma, H., & Manan, H. (2019). Effect of estrogen hormone, 17 β -estradiol on feminisation of banana shrimp, *Penaeus merguensis* (de Man, 1888) postlarvae and the identification of the age of external sex differentiation. *Aquaculture Reports*, 13, Article 100177. <https://doi.org/10.1016/j.aqrep.2018.100177>
- Isa, S. H., Ramlee, M. N. A., Lola, M. S., Ikhwanuddin, M., Azra, M. N., Abdullah, M. T., Zakaria, S., & Ibrahim, Y. (2021). A system dynamics model for analysing the eco-aquaculture system of integrated aquaculture park in Malaysia with policy recommendations. *Environment, Development and Sustainability*, 23(1), 511-533. <https://doi.org/10.1007/s10668-020-00594-4>
- Ismail, M. M., & Abdullah, A. M. (2013). Shrimp trade competitiveness of Malaysia and selected ASEAN countries. *Journal of International Food & Agribusiness Marketing*, 25(1), 98-115. <https://doi.org/10.1080/08974438.2013.800009>
- Kasan, N. A., Ghazali, N. A., Ikhwanuddin, M., & Ibrahim, Z. (2017). Isolation of potential bacteria as inoculum for biofloc formation in Pacific whiteleg shrimp, *Litopenaeus vannamei* culture ponds. *Pakistan Journal of Biological Sciences*, 20(6), 306-313. <https://doi.org/10.3923/pjbs.2017.306.313>
- Kawamura, G., Yong, A. S. K., Loong, A. H., Doison, A., Ooi, S. Y., & Lim, L. S. (2019). Malaysian herbs as feeding attractants and enhancers for the giant freshwater prawn (*Macrobrachium rosenbergii*) and the whiteleg shrimp (*Litopenaeus vannamei*). *Borneo Journal of Marine Science and Aquaculture*, 3(2), 57-67. <https://doi.org/10.51200/bjomsa.v3i2.1793>
- Khai, N. X., Noh, K. M., Islam, G. N., & Ismail, M. M. (2011). *Mangrove conservation and shrimp farming in market* (IKDPM Occasional Paper 2011/16). Institute for Agricultural and Food Policy Studies, Universiti Putra Malaysia.
- Kua, B. C., Fariduddin, M. O., Marzukhi, O., & Iftikhar, A. M. A. (2018). Mortality outbreaks in whiteleg shrimp (*Penaeus vannamei* Bonne 1931) cultured in Peninsular Malaysia. *Asian Fisheries Science*, 31, 242-256. <https://doi.org/10.33997/j.afs.2018.31.S1.017>
- Kumar, V., Roy, S., Behera, B. K., Bossier, P., & Das, B. K. (2021). Acute hepatopancreatic necrosis disease (AHPND): Virulence, pathogenesis and mitigation strategies in shrimp aquaculture. *Toxins*, 13(8), Article 524. <https://doi.org/10.3390/toxins13080524>
- Lee, W. C. (2020). Marketing margins of aquaculture shrimp production in Kedah. *Borneo Journal of Marine Science and Aquaculture*, 4(1), 20-23. <https://doi.org/10.51200/bjomsa.v4i1.1992>
- Levy, T., & Sagi, A. (2020). The "IAG-switch"—A key controlling element in decapod crustacean sex differentiation. *Frontiers in Endocrinology*, 11, Article 651. <https://doi.org/10.3389/fendo.2020.00651>

- Lezer, Y., Aflalo, E. D., Manor, R., Sharabi, O., Abilevich, L. K., & Sagi, A. (2015). On the safety of RNAi usage in aquaculture: The case of all-male prawn stocks generated through manipulation of the insulin-like androgenic gland hormone. *Aquaculture*, 435, 157-166. <https://doi.org/10.1016/j.aquaculture.2014.09.040>
- Liao, I. C., & Chien, Y. H. (2011). The Pacific white shrimp, *Litopenaeus vannamei*, in Asia: The world's most widely cultured alien crustacean. In B. Galil, P. Clark, & J. Carlton (Eds.), *In the wrong place - alien marine crustaceans: Distribution, biology and impacts* (Vol. 6, pp. 489-519). Springer. https://doi.org/10.1007/978-94-007-0591-3_17
- Lightner, D. V. (2011). Virus diseases of farmed shrimp in the Western Hemisphere. *Journal of Invertebrate Pathology*, 106(1), 110-130. <https://doi.org/10.1016/j.jip.2010.09.012>
- Manan, H., Ikhwanuddin, M., Shahreza, M. S., Abol-Munafi, A. B., & Fariduddin, M. O. (2012). Triploidy induction using cold shock treatment in banana shrimp, *Penaeus merguensis* (de Man, 1888). *Borneo Science*, 30, 64-69. https://www.researchgate.net/publication/321487615_Triploidy_induction_using_cold_shock_treatment_in_banana_shrimp_Penaeus_merguensis_De_Man_1888
- Manan, H., Moh, J. H. Z., Kasan, N. A., Suratman, S., & Ikhwanuddin, M. (2017). Identification of biofloc microscopic composition as the natural bioremediation in zero water exchange of Pacific white shrimp, *Penaeus vannamei*, culture in closed hatchery system. *Applied Water Science*, 7, 2437-2446. <https://doi.org/10.1007/s13201-016-0421-4>
- Manan, H., Amin-Safwan, A., Kasan, N. A., & Ikhwanuddin, M. (2020). Effects of biofloc application on survival rate, growth performance and specific growth rate of Pacific whiteleg shrimp, *Penaeus vannamei* culture in closed hatchery system. *Pakistan Journal of Biological Sciences*, 23(12), 1563-1571. <https://doi.org/10.3923/pjbs.2020.1563.1571>
- Manan, H., & Ikhwanuddin, M. (2021). Triploid induction in penaeid shrimps aquaculture: A review. *Reviews in Aquaculture*, 13(1), 619-631. <https://doi.org/10.1111/raq.12489>
- Manan, H., Rosland, N. A., Mat Deris, Z., Che Hashim, N. F., Kasan, N. A., Ikhwanuddin, M., Suloma, A., & Fauzan, F. (2022). 16S rRNA sequences of *Exiguobacterium* spp. bacteria dominant in a biofloc pond cultured with whiteleg shrimp, *Penaeus vannamei*. *Aquaculture Research*, 53(5), 2029-2041. <https://doi.org/10.1111/are.15731>
- Market Research Report. (2022). *Shrimp market size*. Fortune Business Insights Publishers.
- Mazuki, H., & Subramanian, K. (2005). *Shrimp farming in Malaysia*. Agricultural Science and Technology Information (AGRI), Food and Agriculture Organisation of the United Nations.
- Memon, A. J., Ikhwanuddin, M., Talpur, A. D., Khan, M. I., Fariduddin, M. O., Safiah, J., & Abol-Munafi, A. B. (2012). To determine the efficiency of different fresh diets in improving the spermatophore quality of banana shrimp *Penaeus merguensis* (De Man, 1888). *Journal of Animal and Veterinary Advances*, 11(9), 1478-1485. <https://doi.org/10.3923/javaa.2012.1478.1485>
- Mohamad, N., Manan, H., Sallehuddin, M., Musa, N., & Ikhwanuddin, M. (2020). Screening of lactic acid bacteria isolated from giant freshwater prawn (*Macrobrachium rosenbergii*) as potential probiotics. *Aquaculture Reports*, 18, Article 100523. <https://doi.org/10.1016/j.aqrep.2020.100523>
- Mohd-Haikal, I., & Mohammad-Mustafizur, R. (2019). *Status of shrimp production in Malaysia with an emphasis on overexploited species*. Paper presented at the International Conference on Oceanography and Sustainable Marine Production (ICOSMAP), Kuantan, Pahang. <http://irep.iium.edu.my/78587/>

- Muthukrishnan, S., Defoirdt, T., Ina-Salwany, M. Y., Yusoff, F. M., Shariff, M., Ismail, S. I., & Natrah, I. (2019). *Vibrio parahaemolyticus* and *Vibrio harveyi* causing acute hepatopancreatic necrosis disease (AHPND) in *Penaeus vannamei* (Boone, 1931) isolated from Malaysian shrimp ponds. *Aquaculture*, 511, Article 734227. <https://doi.org/10.1016/j.aquaculture.2019.734227>
- Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C. M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., & Troell, M. (2000). Effect of aquaculture on world fish supplies. *Nature*, 405, 1017-1024. <https://doi.org/10.1038/35016500>
- Padilah, B., Rohaiza-Asmini, Y., Gan, H. M., Rozana, W. A. W., Sajiri, W. M. H. W., & Kua, B. C. (2022). Detection of PirA/B toxin genes for acute hepatopancreatic necrosis disease (AHPND) and *Vibrio parahaemolyticus* in *Penaeus vannamei* culture from major white shrimp producing farms in Malaysia. *Pertanika Journal of Tropical Agricultural Science*, 45(1), 171-186. <https://doi.org/10.47836/pjtas.45.1.10>
- Pazir, M. K., Ahmadi, A., & Khezri, P. H. (2022). The effect of COVID-19 pandemic on the shrimp industry of Iran. *Marine Policy*, 136, Article 104900. <https://doi.org/10.1016/j.marpol.2021.104900>
- Rahman, M. S., Majumder, M. K., Sujan, M. H. K., & Manjira, S. (2021). Livelihood status of coastal shrimp farmers in Bangladesh: Comparison before and during COVID-19. *Aquaculture Reports*, 21, Article 100895. <https://doi.org/10.1016/j.aqrep.2021.100895>
- Reardon, T., Mishra, A., Nuthalapati, C. S., Bellemare, M. F., & Zilberman, D. (2020). COVID-19's disruption of India's transformed food supply chains. *Economic and Political Weekly*, 55(18), 18-22. <https://www.gmf.ac.in/wp-content/uploads/2020/08/MAY-2-2020-Vol-LV-No-18.pdf#page=18>
- Rebecca, A. A., & Bhavan, P. S. (2014). Growth promotion and survival enhancement of freshwater prawn *Macrobrachium rosenbergii* postlarvae fed with *Allium sativum*, *Zingiber officinale* and *Curcuma longa*. *International Journal of Pure and Applied Zoology*, 2(2), 138-149.
- Sagi, A., & Aflalo, E. D. (2005). The androgenic gland and monosex culture of freshwater prawn *Macrobrachium rosenbergii* (De Man): A biotechnological perspective. *Aquaculture Research*, 36(3), 231-237. <https://doi.org/10.1111/j.1365-2109.2005.01238.x>
- Sagi, A., & Cohen, D. (1990). Growth, maturation and progeny of sex reversed *Macrobrachium rosenbergii* males. *World Aquaculture*, 21, 87-90.
- Samah, R. (2020). Increasing the fish production with good aquaculture practices. *International Journal of Business and Technology Management*, 2(1), 27-33.
- Shariff, M., & Subasinghe, R. P. (1993). Diseases in Malaysian aquaculture. In R. P. Subasinghe & M. Shariff (Eds.), *Diseases in aquaculture: The current issues* (pp. 49-59). Malaysian Fisheries Society.
- Soo, T. C. C., Devadas, S., Mohamed Din, M. S., & Bhassu, S. (2019). Differential transcriptome analysis of the disease tolerant Madagascar-Malaysia crossbred black tiger shrimp, *Penaeus monodon* hepatopancreas in response to acute hepatopancreatic necrosis disease (AHPND) infection: Inference on immune gene response and interaction. *Gut Pathogens*, 11, Article 13. <https://doi.org/10.1186/s13099-019-0319-4>
- Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., Vlak, J. M., Jones, B., Morado, F., Moss, S., Lotz, J., Bartholomay, L., Behringer, D., Hauton, C., & Lightner, D. V. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of Invertebrate Pathology*, 110(2), 141-157. <https://doi.org/10.1016/j.jip.2012.03.013>

- Sumner, A., Hoy, C., & Ortiz-Juarez, E. (2020). *Estimates of the impact of COVID-19 on global poverty*. United Nations University. <https://doi.org/10.35188/UNU-WIDER/2020/800-9>
- Syahidah, A., Saad, C. R., Daud, H. M., & Abdelhadi, Y. M. (2015). Status and potential of herbal applications in aquaculture: A review. *Iranian Journal of Fisheries Sciences*, 14(1), 27-44.
- Tan, K., Jiang, H., Jiang, D., & Wang, W. (2020). Sex reversal and the androgenic gland (AG) in *Macrobrachium rosenbergii*: A review. *Aquaculture and Fisheries*, 5(6), 283-288. <https://doi.org/10.1016/j.aaf.2019.11.004>
- Taw, N., Thong, P. Y., Ming, L. T., Thanabatra, C., & Salleh, K. Z. (2011). Malaysia shrimp farm redesign successfully combines biosecurity, biofloc technology. *Global Aquaculture Advocate*, 1-6.
- Taw, N. (2013). *Recent developments of biosecurity and biofloc technology*. Paper presented at the Aquaculture America Conference, Nashville, Tennessee.
- Taw, N. (2017). Sustainable shrimp farming in bio secure RAS and biofloc technology: Commercial experience and approaches to disease control. *Journal of Fisheries and Livestock Production*, 5(2), Article 1000231.
- Thean, L. G. (2014). *Efficiency performance of Malaysian brackish water white shrimp* [Master's thesis, Universiti Putra Malaysia]. Institutional Repository.
- Thiang, E. L., Lee, C. W., Takada, H., Seki, K., Takei, A., Suzuki, S., Wang, A., & Bong, C. W. (2021). Antibiotic residues from aquaculture farms and their ecological risks in Southeast Asia: A case study from Malaysia. *Ecosystem Health and Sustainability*, 7(1), Article 1926337. <https://doi.org/10.1080/20964129.2021.1926337>
- Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., & Lightner, D. V. (2013). Determination of the infectious nature of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic Organisms*, 105(1), 45-55. <https://doi.org/10.3354/dao02621>
- Vaseeharan, B., & Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Letters in Applied Microbiology*, 36(2), 83-87. <https://doi.org/10.1046/j.1472-765X.2003.01255.x>
- Vergel, J. C. V. (2017). Current trends in the Philippines' shrimp aquaculture industry: A booming blue economy in the Pacific. *Oceanography & Fisheries Open Access Journal*, 5(4), 85-89. <https://doi.org/10.19080/OFOAJ.2017.05.555668>
- Waiho, K., Fazhan, H., Ishak, S. D., Kasan, N. A., Liew, H. J., Norainy, M. H., & Ikhwanuddin, M. (2020). Potential impacts of COVID-19 on the aquaculture sector of Malaysia and its coping strategies. *Aquaculture Reports*, 18, Article 100450. <https://doi.org/10.1016/j.aqrep.2020.100450>
- Wang, Y. G., Lee, K. L., Najiah, M., Shariff, M., & Hassan, M. D. (2000). A new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon* and its comparison with white spot syndrome (WSS) caused by virus. *Diseases of Aquatic Organisms*, 41(1), 9-18. <https://doi.org/10.3354/dao041009>
- Yan, C. Z. Y., Austin, C. M., Ayub, Q., Rahman, S., & Gan, H. M. (2019). Genomic characterisation of *Vibrio parahaemolyticus* from Pacific white shrimp and rearing water in Malaysia reveals novel sequence types and structural variation in genomic regions containing the Photorhabdus insect-related (Pir) toxin-like genes. *FEMS Microbiology Letters*, 366(17), Article fnz211. <https://doi.org/10.1093/femsle/fnz211>

Short Communication

SSR Markers for Verifying Crosses and Assessing Whitefly Resistance in Soybean Breeding Programme

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ABSTRACT

Selection of hand-pollinated seeds is a critical phase in the breeding programme of whitefly-resistant soybean. This study aimed to verify true crosses and assess whitefly resistance in soybean plants derived from crosses between a superior variety and whitefly-resistant germplasm using SSR markers. The whitefly-sensitive variety Anjasmoro was crossed with IAC 100 and G100H germplasm, serving as donors of resistance genes. Whitefly resistance in the three parental lines and 46 F₁ progenies was evaluated in a greenhouse using a free-choice host test. True crosses were verified through molecular selection in the laboratory using the SSR marker Satt045. The results revealed significant differences in the intensity of whitefly-induced leaf damage among the tested genetic materials. The worst leaf damage, up to 84.7%, was shown by Anjasmoro, while germplasm IAC 100 and G100H showed no leaf damage. The intensity of leaf damage in the offspring of Anjasmoro × IAC 100 and Anjasmoro × G100H varied from 0 to 84.7%. It is hypothesised that no successful cross-pollination occurred between IAC 100 or G100H and Anjasmoro in the plants derived from unsuccessful crosses that exhibited severe leaf damage. Selection using SSR markers indicated that plants with high leaf damage intensity had the same banding pattern as Anjasmoro. In contrast, plants with

low leaf damage intensity exhibited a banding pattern that combined those of Anjasmoro with IAC 100 or Anjasmoro with G100H. These findings demonstrate that SSR markers, supported by greenhouse resistance tests, are reliable for verifying true crosses in soybean breeding programmes targeting whitefly resistance.

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INTRODUCTION

The whitefly (*Bemisia tabaci* Genn.), a polyphagous insect, is a major limiting factor in soybean production in Indonesia. Severe infestations during the dry season can lead to yield losses of up to 80%, even resulting in total crop failure. This leaf-sucking pest causes chlorosis on the host plant due to its activity of extracting phloem sap from the leaves (Zaidi et al., 2017). The honeydew excreted during the feeding process provides an ideal growth medium for sooty mould fungi, which indirectly damages the plant by disrupting the photosynthesis process (Oliveira et al., 2001). The damage caused by whitefly is further exacerbated by its role as a vector of various viral diseases (Jones 2003; Navas-Castillo et al., 2011).

One environmentally friendly method for controlling whitefly is by planting resistant varieties. The process of developing resistant varieties requires a lengthy process that begins with finding the source of resistance genes. The soybean lines IAC 100 and G100H are known to possess resistance to whitefly infestations. Pinheiro et al. (2005) mentioned that the IAC 100 line is a soybean germplasm that has resistance to several main pests on soybean. Meanwhile, the G100H line is the offspring of the cross of IAC 100 with the Hymmeshirazu soybean variety (Sari & Sulisty, 2018). In Indonesia, the Anjasromo soybean variety is widely favoured by farmers due to its large seeds and high productivity, making it a popular choice for cultivation (Krisdiana 2014). However, despite its advantages, Anjasromo is vulnerable to whitefly infestations, posing a challenge for soybean growers in maintaining its yield potential (Sulisty & Inayati, 2016). Therefore, to enhance its resistance, hybridising Anjasromo with IAC 100 and G100H could be a strategic approach, combining the strengths of high yield and large seeds with improved pest resistance.

The next stage is to cross the soybean variety with the resistance gene donor line. The main problem in hand pollination of soybean is the low percentage of successful crossing due to the small size of soybean flowers. In addition, the crossed seeds do not necessarily receive the resistance genes, as soybean is a self-pollinating plant. The expression of resistance genes in the crossed seeds will only be seen after the plants are planted and attacked by the target pest. Therefore, a reliable method is needed to assist soybean breeders in distinguishing between crossed seeds that have received the resistance gene and those that have not. One effective method to assist in distinguishing crossed seeds is the use of molecular markers.

Molecular markers are DNA sequences that are evenly distributed across an organism's genome and are essential for identifying genetic variations among individuals or species (Oliveira & Azevedo, 2022). Among the different types of molecular markers, Simple Sequence Repeat (SSR) markers are widely utilised. SSR markers have proven to be effective tools for analysing genetic diversity, particularly in soybean research (Tasma et al., 2018).

Shakil et al. (2015) highlighted the efficiency of SSR in distinguishing soybean accessions with high genetic similarity. These markers are valued for their codominant inheritance, high polymorphism, reproducibility, widespread distribution across the plant genome, and compatibility with standard Polymerase Chain Reaction (PCR) techniques (Amiteye, 2021; Singh & Singh, 2018). Although no specific SSR primers have yet been reported to be directly associated with whitefly resistance in soybean, previous studies have shown that the SSR marker Satt045 displays high polymorphism and is frequently used to assess genetic distance among soybean germplasm (Chotiyarnwong et al., 2007; Dyah et al., 2024; Meesang et al., 2001) and to support selection processes in breeding programmes (Tasma et al., 2018). The objective of this study was to verify true crosses and assess whitefly resistance in F₁ plants derived from crosses between a high-yielding soybean variety and whitefly-resistant germplasm using the SSR marker Satt045.

METHODS

Plant Genetic Material

The soybean seeds tested were F₁ progenies from a cross between the Anjasmoro variety and the IAC 100 and G100H lines. Anjasmoro is a superior variety widely cultivated by farmers in Indonesia. Released by the Ministry of Agriculture of the Republic of Indonesia in 2001, this variety boasts a high yield potential of up to 2.25 tons per hectare and large seed size (15.3 g per 100 seeds). However, Anjasmoro has a weakness, which is its susceptibility to whitefly attacks (Sulistyo & Inayati, 2016). Meanwhile, IAC 100 and G100H are soybean germplasms that exhibit resistance to whitefly (Pinheiro et al., 2005; Sari & Sulistyo, 2018).

Greenhouse Experiment

The study was conducted in the greenhouse of Indonesian Legume and Tuber Crops Research Institute (ILETRI), East Java, Indonesia. The experiment involved three groups of plant materials consisting of parental lines and their F₁ progenies. The parental lines (Anjasmoro, IAC 100, and G100H) served as reference controls, while the F₁ progenies were derived from two different crosses: Anjasmoro × IAC 100 and Anjasmoro × G100H. This grouping allowed clear comparison and ensured consistent evaluation of whitefly resistance among genotypes.

All soybean seeds were planted in polybags containing a 1:1 mixture of soil and manure. Each seed from one pod was planted in one polybag. Urea, SP36, and KCl fertilisers were applied at planting time at rates equivalent to 50, 100, and 100 kg per hectare, respectively. Resistance testing against whitefly was conducted using a free-choice test method. The whitefly infestation followed the method used by Mansaray and Sundufu (2009), in which 10 adult insects were placed on the leaf surface of each individual soybean plant at two weeks of age.

Damage to the fifth trifoliate leaf from the growing point due to whitefly attack was assessed when the plants were seven weeks old. Leaf damage was scored visually using the method of Sari and Sulistyo (2018), applying a 0-4 rating scale as described in Table 1.

The percentage of leaf damage intensity (LDI) was then calculated based on the visual scores using the following Equation 1:

$$LDI (\%) = \frac{\sum(n_i \times v_i)}{N \times V} \times 100\% \tag{1}$$

When n_i is the number of leaves assigned a score v_i , N is the total number of leaves observed, and V is the maximum score value. This formula converts the qualitative scoring data into quantitative values representing the degree of whitefly damage. Plant resistance levels were then categorised based on the method of Chiang and Talekar (1980), as presented in Table 2.

Table 1
Scoring scale and description of leaf damage symptoms due to whitefly infestation

Score	Description of Symptoms	Severity Level
0	No visible symptoms; leaves appear healthy without curling or sooty mould.	No damage
1	Mild symptoms: leaf curling and/or sooty mould on $\leq 25\%$ of leaf surface.	Slight
2	Moderate symptoms; leaf curling and/or sooty mould on 25-50% of leaf surface.	Moderate
3	Severe symptoms: leaf curling and/or sooty mould covering 50-75% of leaf surface; pod and seed development are abnormal.	Severe
4	Very severe symptoms; leaf curling and/or sooty mould on $>75\%$ of leaf surface; pod and seed development failure.	Very severe

Table 2
Classification criteria for determining resistance levels to whitefly based on mean (X) and standard deviation (SD)

Resistance Category	Criteria
Highly Resistant (HR)	$X_i \leq (X - 2SD)$
Resistant (R)	$(X - 2SD) \leq X_i \leq (X - SD)$
Moderately Susceptible (MS)	$(X - SD) \leq X_i \leq X$
Susceptible (S)	$X \leq X_i \leq (X + 2SD)$
Highly Susceptible (HS)	$X_i \geq (X + 2SD)$

Note. X is the population mean; X_i is the individual observed value; and SD is the standard deviation of the population

Genomic DNA Isolation

Soybean genomic DNA was isolated from 0.5 g of leaf samples from three-week-old plants using a modified Doyle and Doyle (1990) method, which included the addition of 2% (w/v) PVP. The resulting DNA pellets were dissolved in 100 µL of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and treated with 2 µL of 10 mg/mL RNase (Invitrogen, USA), then incubated at 37°C for 1 hour. The quality of the genomic DNA was assessed through electrophoresis on a 1% agarose gel, and the results were observed under UV light using a UV Transilluminator (UVP, UK). Quantitative analysis of the genomic DNA was performed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

SSR Marker Analysis

The SSR marker used in this study was Satt045. The primer sequences were as follows: 5'TGGTTTCTACTTTCTATAATTATTT 3' and 5' ATGCCTCTCCCTCCT 3' for the forward and reverse primers, respectively (Cregan et al., 1999). The repeat type of the SSR marker is (AAT)₁₈, located on chromosome 15, and its PCR product size is 139 bp.

PCR amplification of the SSR marker was performed using a Biometra PCR machine using genomic DNA of the parents and each of the F₁ progenies as DNA templates. The genomic DNA of each hybrid plant was amplified using a 10 µL volume in a reaction mixture containing 2 µL of 20 ng template DNA, 5 µL of Kapa2G Fast Ready Mix (KAPA Biosystems, USA), 0.5 µL each of 10 µM forward and reverse primers, and 2 µL of sterile ddH₂O. The PCR protocol, as described by Tasma et al. (2001; 2018), involved an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of DNA amplification with denaturation at 94°C for 30 seconds, primer annealing at 55°C for 1 minute, DNA extension at 72°C for a minute, post-extension at 60°C for 15 minutes, and a final incubation at 10°C for 4 minutes.

The PCR products were then analysed by electrophoresis using an 8% polyacrylamide gel in a vertical electrophoresis tank filled with 1x Tris Borate EDTA (TBE) buffer, running at 90 volts for 115 minutes. Visualisation of DNA bands was done under a UV Transilluminator Gel Doc (Bio Rad, California, USA) using the ethidium bromide staining method.

RESULTS AND DISCUSSION

Leaf Damage Intensity

The resistance testing against whitefly indicated that the Anjasmoro variety experienced the most severe leaf damage, reaching 84.7% (Table 3). This finding is consistent with the research by Sulistyo and Inayati (2016), which reported leaf damage in the Anjasmoro variety at 76.6% in host choice tests.

Additionally, Krisnawati et al. (2023) stated that Anjasmoro remains classified as a soybean variety susceptible to whitefly, regardless of insecticide treatment. In contrast, the IAC 100 and G100H showed no significant leaf damage during testing. This result differs slightly from the findings of Sari and Sulisty (2018), who reported leaf damage in G100H ranging from 10.91% to 18.03%. Krisnawati et al. (2023) also reported that leaf damage in G100H reached 5.69% and 9.87% when treated and untreated with insecticides, respectively. Nevertheless, both germplasms are still categorised as soybean varieties resistant to whitefly.

The resistance evaluation of the Anjasmoro $\times$ IAC 100 progeny against whitefly infestation showed significant variations in leaf damage, ranging from 0% to 84.7%, with an average of 18.1% (Table 3). Based on the degree of leaf damage, the Anjasmoro $\times$ IAC 100 progeny were classified into four resistance groups: nine highly resistant plants, nine resistant plants, seven susceptible plants, and four highly susceptible plants. The highly resistant group (plants numbered 7, 8, 9, 12, 14, 15, 16, 17, and 18) exhibited no leaf damage. The resistant group (plants numbered 3, 4, 5, 6, 10, 11, 13, 25, and 27) showed leaf damage between 4.5% and 16.7%, which is below the population average. In contrast, the susceptible and highly susceptible groups (plants numbered 1, 2, 19, 20, 21, 22, 23, 24, 26, 28, and 29) experienced more severe leaf damage, ranging from 19.7% to 84.7%.

These findings suggest that the resistant and highly resistant groups may have acquired resistance genes from the IAC 100 germplasm, as evidenced by the relatively low leaf damage. Conversely, the heightened leaf damage observed in the susceptible and highly susceptible groups implies a potential failure in hand pollination, leading to the absence of resistance gene transmission from IAC 100 to the Anjasmoro variety.

The resistance testing results on the Anjasmoro $\times$ G100H progeny revealed slight disparities compared to those observed in the Anjasmoro $\times$ IAC 100 progeny. The resistance evaluation of Anjasmoro $\times$ G100H progeny indicated leaf damage ranging from 0% to 50.3% (Table 3). Although the leaf damage was less severe compared to the Anjasmoro $\times$ IAC 100 progeny, only a limited number of plants in the Anjasmoro $\times$ G100H progeny exhibited resistance to whitefly infestation. Based on the extent of leaf damage, the Anjasmoro $\times$ G100H progeny were categorised into four resistance groups: three highly resistant plants (plant numbered 11, 16 and 17), six moderately susceptible plants (plant numbered 9, 10, 12, 13, 14, and 15), seven susceptible plants (plant numbered 1, 2, 4, 5, 6, 7, and 8) and one highly susceptible plant (plant numbered 3). The moderately susceptible, susceptible, and highly susceptible groups exhibited leaf damage ranging from 10.0% to 50.3%. The predominance of the latter three groups in the tested Anjasmoro $\times$ G100H progeny suggests that hand pollination in the crossbreeding of Anjasmoro with G100H might be more challenging, indicating a difficulty in transmitting resistance genes from G100H to Anjasmoro.

Table 3
Leaf damage assessment and resistance categories of Anjasmoro, IAC 100, G100H and their progenies in greenhouse conditions

Genotype	Leaf damage intensity (%)	Resistance category	Genotype	Leaf damage intensity (%)	Resistance category
Anj/IAC100-1	20.0	S	Anj/G100H-1	17.9	S
Anj/IAC100-2	33.0	S	Anj/G100H-2	33.3	S
Anj/IAC100-3	10.4	R	Anj/G100H-3	50.3	HS
Anj/IAC100-4	5.0	R	Anj/G100H-4	41.7	S
Anj/IAC100-5	4.2	R	Anj/G100H-5	25.0	S
Anj/IAC100-6	15.0	R	Anj/G100H-6	36.5	S
Anj/IAC100-7	0	HR	Anj/G100H-7	20.0	S
Anj/IAC100-8	0	HR	Anj/G100H-8	20.8	S
Anj/IAC100-9	0	HR	Anj/G100H-9	15.0	MS
Anj/IAC100-10	14.8	R	Anj/G100H-10	10.0	MS
Anj/IAC100-11	4.5	R	Anj/G100H-11	0	HR
Anj/IAC100-12	0	HR	Anj/G100H-12	10.0	MS
Anj/IAC100-13	15.0	R	Anj/G100H-13	8.3	MS
Anj/IAC100-14	0	HR	Anj/G100H-14	15.0	MS
Anj/IAC100-15	0	HR	Anj/G100H-15	10.0	MS
Anj/IAC100-16	0	HR	Anj/G100H-16	0	HR
Anj/IAC100-17	0	HR	Anj/G100H-17	0	HR
Anj/IAC100-18	0	HR	Anjasmoro	84.7	HS
Anj/IAC100-19	84.7	HS	G100H	0	HR
Anj/IAC100-20	62.5	HS	Mean	17.4	
Anj/IAC100-21	63.4	HS	Std. Dev.	15.02	
Anj/IAC100-22	22.1	S			
Anj/IAC100-23	60.9	HS			
Anj/IAC100-24	21.3	S			
Anj/IAC100-25	16.7	R			
Anj/IAC100-26	24.4	S			
Anj/IAC100-27	15.4	R			
Anj/IAC100-28	19.7	S			
Anj/IAC100-29	31.3	S			
Anjasmoro	84.7	HS			
IAC 100	0	HR			
Mean	18.1				
Std. Dev.	22.12				

Note. HR = highly resistant; R = resistant; MS = moderately susceptible; S = susceptible; HS = highly susceptible

SSR Evaluation

The Satt045 primer used in this study generated polymorphic band patterns ranging in size from 125 to 200 base pairs (bp) (Figure 1). These findings are consistent with those reported by Chotiyarnwong et al. (2007), who observed polymorphism using the Satt045 primer in 160 soybean genotypes in Thailand, with band sizes ranging from 122 to 157 bp. Additionally, Dyah et al. (2024) identified that the Satt045 primer could produce 42 alleles with sizes ranging from 138 to 202 bp. In this study, the three soybean parental lines were distinguishable based on their banding patterns. Specifically, the Anjasmoro variety exhibited band patterns ranging from 136 to 178 bp, the IAC 100 germplasm displayed band patterns ranging from 125 to 160 bp, and the G100H germplasm showed band patterns ranging from 127 to 160 bp (Figure 1).

SSR marker analysis on the progeny population from the cross between Anjasmoro and IAC 100 showed that plants classified as susceptible (plants numbered 1, 2, 22, 24, 26, 28, and 29) and highly susceptible (plants numbered 19, 20, 21, and 23) exhibited banding patterns identical to Anjasmoro. This observation supports the hypothesis that hand pollination was unsuccessful, preventing the transfer of resistance genes from IAC 100 to Anjasmoro. In contrast, the resistant plants (numbered 3, 4, 5, 6, 10, 11, and 13) and highly resistant plants (numbered 7, 8, 9, 12, 14, 15, 16, 17, and 18) exhibited combined banding patterns from both IAC 100 and Anjasmoro, indicating successful gene transfer for resistance from IAC 100 to Anjasmoro. Furthermore, the SSR marker Satt045 effectively distinguished true F₁ progeny by identifying plants with combined banding patterns reflective of both parental genotypes.

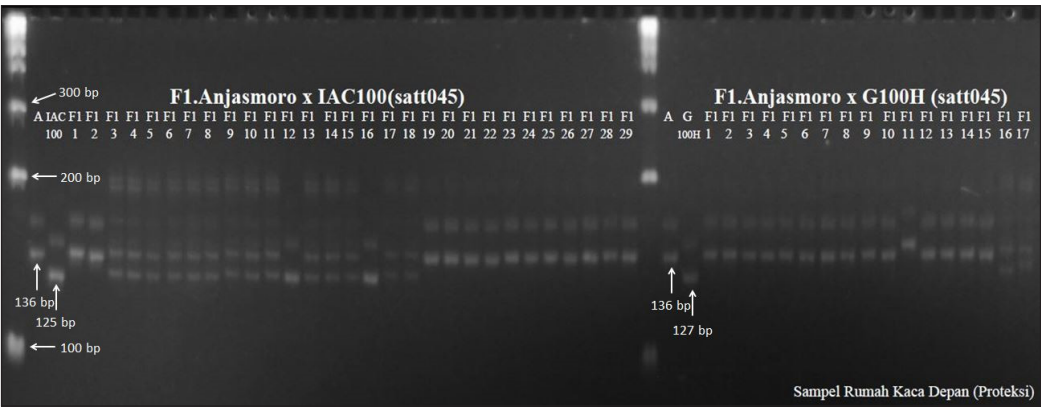


Figure 1. SSR marker banding patterns of Anjasmoro, IAC 100, G100H, and their progenies

Significant findings were observed in plants numbered 25 and 27 from the Anjasmoro $\times$ IAC 100 cross. Greenhouse resistance assessments revealed minimal leaf damage caused by whitefly infestation in these plants, indicating that they could be categorised as resistant. However, SSR marker analysis showed that both plants had identical banding patterns to Anjasmoro, suggesting they were selfed progeny rather than true hybrids. This underscores the utility of SSR markers in assisting soybean breeders to effectively screen out undesired progeny resulting from crosses. The ability of SSR markers to produce clear codominant segregation patterns, such as the observed 1:2:1 ratio in the F₂ populations, enables breeders to reliably distinguish true heterozygotes from homozygous individuals (Cregan et al., 1999; Singh & Singh, 2018; Tasma et al., 2018). This precision is crucial for eliminating undesired progeny and accelerating the development of improved soybean lines.

SSR marker analysis of the offspring resulting from the Anjasmoro $\times$ G100H cross revealed that all plants categorised as moderately susceptible, susceptible, and highly susceptible exhibited banding patterns identical to those of Anjasmoro. In contrast, plants classified as highly resistant (plants numbered 11, 16, and 17) displayed combined banding patterns from both Anjasmoro and G100H. These findings underscore the effectiveness of SSR markers in reinforcing conventional selection methods conducted under controlled greenhouse conditions. Specifically, the SSR marker Satt045 proved highly reliable in differentiating true F₁ progeny by identifying the presence of resistance alleles contributed by G100H.

CONCLUSION

Greenhouse resistance testing of Anjasmoro, IAC 100, G100H, and their progenies revealed that some hybrid plants derived from these parental lines exhibited resistance to whitefly infestation, as indicated by relatively low levels of leaf damage. Selection using the SSR marker Satt045 confirmed that plants with low leaf damage exhibited banding patterns combining features from Anjasmoro with either IAC 100 or G100H. In contrast, hybrid plants with high levels of leaf damage displayed banding patterns identical to Anjasmoro. These findings highlight the reliability of the SSR marker Satt045 in verifying true F₁ hybrids and its effectiveness as a molecular tool for supporting resistance selection in soybean breeding programmes.

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REFERENCES

- Amiteye, S. (2021). Basic concepts and methodologies of DNA marker systems in plant molecular breeding. *Heliyon*, 7(10), Article e08093. <https://doi.org/10.1016/j.heliyon.2021.e08093>
- Chiang, H. S., & Talekar, N. S. (1980). Identification of source of resistance to the beanfly and two other Agromyzid flies in soybean and mungbean. *Journal of Economic Entomology*, 73(2), 197-199. <https://doi.org/10.1093/jee/73.2.197>
- Chotiarnwong, O., Chatwachirawong, P., Chanprame, S., & Srinives, P. (2007). Evaluation of genetic diversity in Thai indigenous and recommended soybean varieties by SSR markers. *Thai Journal of Agricultural Science*, 40(3-4), 119-126.
- Cregan, P. B., Jarvik, T., Bush, A. L., Shoemaker, R. C., Lark, K. G., Kahler, A. L., Kaya, N., VanToai, T. T., Lohnes, D. G., Chung, J., & Specht, J. E. (1999). An integrated genetic linkage map of the soybean genome. *Crop Science*, 39(5), 1464-1490. <https://doi.org/10.2135/cropsci1999.3951464x>
- Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, 12(1), 13-15.
- Dyah, R. P., Wibisono, K., Terryana, R. T., Nugroho, K., Utari, R., Suparjo, Umar, Lestari, P., & Tasma, I. M. (2024). Diversity analysis of 53 soybean accessions introduced from China based on morphological characteristics and SSR markers. *Current Applied Science and Technology*, 24(3), Article 259116. <https://doi.org/10.55003/cast.2024.259116>
- Jones, D. R. (2003). Plant viruses transmitted by whiteflies. *European Journal of Plant Pathology*, 109(3), 195-219. <https://doi.org/10.1023/A:1022846630513>
- Krisdiana, R. (2014). Penyebaran varietas unggul kedelai dan dampaknya terhadap ekonomi pedesaan [Dissemination of improved soybean varieties and its impact on rural economy]. *Jurnal Penelitian Pertanian Tanaman Pangan*, 33(1), 61-69. <https://doi.org/10.21082/jpntp.v33n1.2014.p61-69>
- Krisnawati, A., Adie, M. M., Krisdiana, R., Prayogo, Y., Soehendi, R., & Baliadi, Y. (2023). Differential levels of soybean resistance to the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) under controlled and uncontrolled environments are associated with plant age, damage intensity, and trichome density. *Bioscience Journal*, 39, Article e39024. <https://doi.org/10.14393/BJ-v39n0a2023-63174>
- Mansaray, A., & Sundufu, A. J. (2009). Oviposition, development and survivorship of the sweet potato whitefly *Bemisia tabaci* on soybean *Glycine max*, and the garden bean *Phaseolus vulgaris*. *Journal of Insect Science*, 9(1), Article 1. <https://doi.org/10.1673/031.009.0101>
- Meesang, N., Ranamukhaarachchi, S. L., Petersen, M. J., & Andersen, S. B. (2001). Soybean cultivar identification and genetic purity analysis using microsatellite DNA markers. *Seed Science and Technology*, 29(3), 637-645.
- Navas-Castillo, J., Fiallo-Olivé, E., & Sánchez-Campos, S. (2011). Emerging virus diseases transmitted by whiteflies. *Annual Review of Phytopathology*, 49(1), 219-248. <https://doi.org/10.1146/annurev-phyto-072910-095235>
- Oliveira, M. R. V., Henneberry, T. J., & Anderson, P. (2001). History, current status, and collaborative research projects for *Bemisia tabaci*. *Crop Protection*, 20(9), 709-723. [https://doi.org/10.1016/S0261-2194\(01\)00108-9](https://doi.org/10.1016/S0261-2194(01)00108-9)

- Oliveira, M., & Azevedo, L. (2022). Molecular markers: An overview of data published for fungi over the last ten years. *Journal of Fungi*, 8(8), Article 803. <https://doi.org/10.3390/jof8080803>
- Pinheiro, J. B., Vello, N. A., Rossetto, C. J., & Zucchi, M. I. (2005). Potential of soybean genotypes as insect resistance source. *Crop Breeding and Applied Biotechnology*, 5(3), 294-301. <https://doi.org/10.12702/1984-7033.v05n03a06>
- Sari, K. P., & Sulisty, A. (2018). Assessment of soybean resistance to whitefly (*Bemisia tabaci*) infestations. *Pertanika Journal of Tropical Agricultural Science*, 41(2), 825-832.
- Shakil, S. K., Sultana, S., Hasan, M. M., Hossain, M. M., Ali, M. S., & Prodhan, S. H. (2015). SSR marker based genetic diversity analysis of modern rice varieties and coastal landraces in Bangladesh. *Indian Journal of Biotechnology*, 14(1), 33-41.
- Singh, B. D., & Singh, A. K. (2018). *Marker assisted plant breeding: Principles and practices*. Springer. <https://doi.org/10.1007/978-81-322-2316-0>
- Sulisty, A., & Inayati, A. (2016). Mechanisms of antixenosis, antibiosis, and tolerance of fourteen soybean genotypes in response to whiteflies (*Bemisia tabaci*). *Biodiversitas*, 17(2), 447-453. <https://doi.org/10.13057/biodiv/d170207>
- Tasma, I. M., Lorenzen, L. L., Green, D. E., & Shoemaker, R. C. (2001). Mapping genetic loci for flowering time, maturity, and photoperiod insensitivity in soybean. *Molecular Breeding*, 8(1), 25-35. <https://doi.org/10.1023/A:1011998116037>
- Tasma, I. M., Yani, N. P. M. G., Purwaningdyah, R., Satyawan, D., Nugroho, K., Lestari, P., Trijatmiko, K. R., & Mastur, M. (2018). Genetic diversity analysis and F2 population development for breeding of long juvenile trait in soybean. *Jurnal AgroBiogen*, 14(1), 11-22. <https://doi.org/10.21082/jbio.v14n1.2018.p11-22>
- Zaidi, S. S. E. A., Briddon, R. W., & Mansoor, S. (2017). Engineering dual begomovirus-*Bemisia tabaci* resistance in plants. *Trends in Plant Science*, 22(1), 6-8. <https://doi.org/10.1016/j.tplants.2016.11.005>

Effects of Dietary Coffee Ground Supplementation on Carcass Traits, Meat Quality, and Faecal Pathogenic Bacterial Populations in Japanese Quails (*Coturnix coturnix japonica*)

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ABSTRACT

This study aimed to investigate the effects of dietary coffee ground supplementation on the carcass traits, meat quality, and faecal pathogenic bacteria of Japanese quails. A completely randomised design (CRD) was employed using 180 nine-week-old male quails, which were randomly assigned to three treatment groups: a control diet (T1) and control diets supplemented with 10 g/kg (T2) and 20 g/kg (T3) of coffee grounds. Each treatment consisted of five replicates with 12 birds per replicate, all maintained on an *ad libitum* feeding and watering regimen. At the end of the experiment, samples were taken from each treatment for evaluation of carcass traits and meat quality, and samples of faeces were analysed for bacterial populations. The findings revealed that the dietary treatment of 10 and 20 g/kg of coffee grounds significantly ($P<0.05$) increased carcass traits. The percentage of commercial cuts and internal organs was favourably enhanced. Dry matter and crude protein content of breast meat were also increased ($P<0.05$) when the coffee grounds were fed. When fed, the breast meat dry matter and crude protein content were increased, and the yellowness (b^*) of the breast meat and skin improved at 0 and 24 hours post-slaughter. Notably, the 10 and 20 g/kg of coffee ground diets caused a significant ($P<0.01$) decrease of *E. coli* and *Salmonella* spp. in their faeces.

In conclusion, dietary supplementation with 10-20 g/kg of coffee grounds in 15-week-old Japanese quails enhanced carcass yield, improved meat quality, and exerted potent antimicrobial effects by suppressing faecal pathogenic bacteria.

Keywords: coffee ground, carcass traits, Japanese quails, meat quality, pathogenic bacteria

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INTRODUCTION

As the world's population is growing, the need for greater yield in agriculture and animal husbandry to ensure food supply is increasing. In this regard, the increasing demand for high-quality animal protein has highlighted the importance of developing more environmentally friendly and economically viable poultry production systems, especially for Japanese quails (*Coturnix coturnix japonica*). Japanese quails are very important for their fast growth rates, early sexual maturity, large egg production and high feed efficiency. Moreover, they are easily adaptable to various husbandry conditions; have low production costs; are easy to manage; require very little space; and are suitable for sustainable poultry farming (Batoool et al., 2021; Mizutani, 2003; Vali, 2008). All the above benefits notwithstanding, the poultry industry is beset with huge challenges, such as the increasing production costs of animal feeds and the fear of antimicrobial resistance. The universal use of antibiotics as growth promoters has resulted in the emergence of pathogens that are cross-resistant to antibiotics and residues in animal tissues, which present significant health risks to animals and humans. The use of antibiotics as growth promoters has sparked continuous research on natural alternatives to improve animal performance and product quality after the European Union (EU) banned their use as growth promoters in 2006 (Castanon, 2007). At the same time, allocation of agricultural resources for animal feed directly affects the production of human food, hence there is a concern about long-term food security. These factors all call for a need to look at alternative and sustainable feedstuff sources. In recent years, research has been directed to the use of pharmacologically active compounds from medicinal plants.

Research has shown that different herbs, when fed to animals, can enhance animal performance to a great extent (Posan et al., 2023), and combinations of multiple medicinal plants in poultry feed can facilitate growth and strengthen the immune system (Phromnoi et al., 2025). Coffee is a major crop globally and produces several useful products, but it produces a significant amount of waste, which can be an environmental problem. Coffee grounds are a major organic byproduct of the coffee processing that is not used. If not properly handled, it is a source of environmental pollution (Ismail et al., 2014; Nillian et al., 2020). Consequently, it is essential to take environmental protection measures to find alternative applications for such waste materials as a matter of great urgency. Arabica beans are the more delicate flavoured beans, which account for a significant amount of the spent coffee grounds (SCG) produced (Ho et al., 2012; Solange et al., 2011).

Spent coffee grounds (SCG), one of the major byproducts of coffee processing, are a promising and sustainable feed raw material for animal production, especially in terms of environmental and economic benefits. In the global coffee industry, about 70% of the total consumption is *Coffea Arabica*, and 30% is *Coffea Canephora* (Robusta variety), producing millions of tonnes of waste every year (DaMatta et al., 2007; Marcel et al., 2011).

SCG contain various bioactive compounds with potential health benefits for the animal, such as polyphenols, caffeine, vitamins, fibre, and protein, which can improve nutrient utilisation and animal health (Ho et al., 2012; Tajik et al., 2017). Notably, SCG is rich in chlorogenic acids and other active substances with well-established powerful antioxidant and antibacterial activities (Bertrand et al., 2012).

Additionally, coffee contains biologically active fatty acids and triglycerides that have proven anti-inflammatory, anti-bacterial, anti-cancer, anti-diabetic and anti-atherosclerotic properties (Al-Asmari et al., 2020). Incorporation of SCG into animal feed, especially for Japanese quails, can enhance the carcass properties and meat quality, along with gut health. Coffee polyphenols, such as phenolic acids and flavonoids, are known to have beneficial effects on lipid metabolism, antimicrobial activity and antioxidant processes in animal muscle tissues, which positively influence meat quality.

Murwani et al. (2024) also reported that phytogetic compounds, such as those found in SCG, can reduce intestinal inflammation and enhance the productive performance of Japanese quails. In addition to their nutritional benefits, the incorporation of SCG into animal feed contributes to environmental sustainability by valorising agricultural waste materials. This strategy provides a practical and eco-friendly alternative to conventional growth promoters in animal production, while also meeting the growing consumer demand for sustainable poultry products. This study evaluated the effects of dietary coffee ground supplementation on the carcass traits, meat quality, and faecal pathogenic bacteria populations of Japanese quails. We hypothesised that the dietary inclusion of coffee grounds would enhance carcass traits, improve meat quality, and beneficially modulate faecal microbial populations. Ultimately, this research aims to support the utilisation of coffee grounds as a sustainable feed ingredient and an eco-friendly alternative to conventional growth promoters in poultry production.

MATERIALS AND METHODS

Code of Conduct for Laboratory Animals

The current investigation was reviewed by the Institutional Animal Care and Committee, Princess of Naradhiwas University, Thailand, No. PNU.AE-2024/01.009.

Coffee Grounds Preparation

Coffee grounds were collected from Muang Narathiwat district, Narathiwat province. After collection, the samples were sun-dried for two days and subsequently dried in a hot-air oven at 60-65 °C for an additional day. The dried coffee grounds were then finely ground using an electric grinder and passed through a 1-mm mesh sieve to obtain a uniform particle size. The processed coffee ground powder was stored in sealed glass containers and refrigerated at 4°C until use in the experiment.

Experimental Animal and Management

A 6-week feeding trial was carried out on 180 nine-week-old male Japanese quails (9-15 weeks) in a completely randomised design (CRD). The birds that were selected for uniform starting body weight were randomly assigned to three different dietary treatments. Five replicates were used for each treatment, with 12 quails in each replicate, making a total of 60 quails per treatment group. Throughout the experiment period, quails were provided with *ad libitum* access to a feed and drinking water. All birds were kept in an open system, and good hygiene and ventilation were maintained. In this experiment, the experimental diet was a commercial quail starter feed fed during the first rearing period (1-4 weeks). The guaranteed nutritional composition of this feed was 22% crude protein, at least 3% crude fat and a maximum of 13% moisture. Table 1 shows the complete ration composition and detailed nutritional analysis of this commercial quail feed.

Carcass Traits

At the end of the experiment (15 weeks of age), carcass traits were evaluated according to the procedures described by Moreng and Avens (1985). Ten quails from each treatment group (two birds per replicate) were randomly selected, weighed, and manually slaughtered after being fasted for 12 hours to determine carcass traits, including dressing, eviscerated weight, carcass, gizzard, liver, heart, spleen, and other total edible parts, which were weighed and calculated for the quail carcass percentage.

Table 1
Ration composition and nutritional content of the quail commercial feed

Composition	Content (%)
Moisture	13
Crude Protein	22
Crude Fat	3
Ash	13
Calcium	3
Phosphorus	0.62

Meat Quality

Meat quality assessment was performed on the *Pectoralis major* muscle, utilising two boneless, skinless breast fillets collected per replicate. Measurements for pH, colour, and drip loss values were executed in accordance with the methodologies described by Wattanachant (2003) and Wattanachant et al. (2004).

For proximate composition analysis, two breast meat samples per replicate were collected. The percentages of crude protein, crude fat, and dry matter were subsequently determined using the methodology outlined by the Association of Official Analytical Chemists (AOAC) (2000).

pH value analysis: a pH meter (Seven2Go™, Mettler-Toledo, Switzerland) was used to measure the pH of breast meat. The probe METTLER TOLEDO Inlab® 413 IP67 was used and calibrated at 4.0 and 7.0.

For colour analysis, raw, undamaged breast meat fillets (two birds per replicate) were used to measure surface colour using the Complete International Commission on Illumination (CIE) system. Colour parameters measured included lightness (L^*), redness (a^*), and yellowness (b^*). The L^* value represents whiteness (0) to darkness (100); the a^* expression ranges from redness (+) to greenness (-); and the b^* observation denotes yellowness (+) to blueness (-). Meat colour measurements were performed in triplicate on the upper, middle, and lower surfaces of each sample using a Colour Reader CR-13 (Konica Minolta Sensing, Inc., Japan).

To determine water-holding capacity via drip loss, breast meat samples from two quails per replicate were analysed. Fillets were cut into three strips, each measuring 1.5 cm wide, 3.0 cm long, and 0.5 cm thick. Each strip was then individually weighed, placed in a sealed polyethylene bag, and kept at a chilling temperature between 1 and 4 °C for 24 hours. After this period, the samples were reweighed, and the drip loss was calculated as the percentage of weight reduction relative to the initial sample weight.

Faecal Pathogenic Bacteria

At the end of the experimental period, five faecal samples were collected from quails within each treatment group to quantify pathogenic bacterial populations. The analyses for *Escherichia coli* (*E. coli*) and *Salmonella* spp. were conducted using the methodology prescribed by the United States Food and Drug Administration (FDA) (2020).

In the *Escherichia coli* (*E. coli*) population assay, preparing 25 grams of faecal sample, 0.1% peptone water buffer was added to 225 millilitres. They were then homogenised using a stomacher. After that, the samples were diluted in 0.85% normal saline solution to achieve the proper concentrations (ranging from 10^{-1} to 10^{-5}). The diluted samples were placed on the surface of the Chrom ID™ Coli agar plates by the spread plate technique. The plates were incubated for 24 hours at 37 °C. After incubation, the plates were evaluated for the *E. coli* population. The typical colony morphology was observed and recorded, and the number of pink-purple colonies was counted.

For the *Salmonella* spp. population assay, preparing 25 grams of faecal sample, 0.1% peptone water buffer was added to 225 millilitres for a pre-enrichment medium. They were then homogenised using a stomacher. Then, samples were diluted in 0.85% normal saline solution to make proper concentrations at dilution levels ranging from 10^{-1} to 10^{-5} . The diluted samples were placed on the surface of the xylose lysine deoxycholate (XLD) agar plates. Spread the sample evenly on the agar surface using the spread plate technique. The plates were incubated for 24 hours at 37 °C. Following incubation, the plates were evaluated for *Salmonella* spp. population, and the colonies were observed and counted. Colonies will appear as round, medium-sized, and red with a black centre due to hydrogen sulphide production. The colonies' surrounding agar will continue to be red.

Statistical Analysis

All experimental data were subjected to a one-way analysis of variance (ANOVA). When significant differences were detected, treatment means were compared using Duncan's Multiple Range Test (DMRT). Data distribution, normality, and descriptive statistics were verified utilising the UNIVARIATE procedure in SAS software (SAS, 2017).

RESULT AND DISCUSSION

Carcass Traits

The present study assessed the effects of dietary supplementation with coffee grounds, at concentrations of 0, 10, and 20 g/kg, on the carcass traits of 15-week-old Japanese quails. The results revealed that supplementing the diet of Japanese quails with coffee grounds, particularly at 10 g/kg (T2) and 20 g/kg (T3), leads to significant quantitative improvements in growth and carcass yield compared to the control group (Table 2). Specifically, the T2 group showed a 7.02% increase in live body weight (171.95 g vs. 160.67 g) and an 8.79% increase in dressing weight (152.84 g vs. 140.49 g). Eviscerated weight also increased by 7.57% in the T2 group. These improvements indicate that the moderate levels of coffee ground supplementation improved overall development of the birds and a significant increase in actual carcass percentage was noted, which being 88.87% in the coffee ground as compared to 87.44% in the control. In addition, the researchers found that there were significant increases in high-value meat amounts and some physiological parameters without any adverse effects on proportions of abdominal fat or internal organs. The percentage of breast and skin weight increased by 9.41% in the T2 group (28.38% vs. 25.94%), while thigh and drumstick weights saw improvements of 14.03% (12.03% vs. 10.55%) and 10.55% (8.28% vs. 7.49%), respectively. Lastly, there were greater weights of testes in quails fed the 10 g/kg and 20 g/kg coffee grounds groups compared to the control group. However, the supplementation did not significantly affect the percentage of the liver, heart and gizzard, suggesting that it does not affect the relative size of metabolic organs, but rather muscle and skeletal growth. The present study shows that feeding coffee grounds in the diet of Japanese quails considerably improves the carcass characteristics. It is noted that dressing and eviscerated weights improved, while the percent of the carcass, breast meat, thigh, drumstick, wing, shank, spleen and testis improved. The improvements are said to be due to the wide range of bioactive compounds found in coffee grounds that have strong antioxidant properties. This finding aligns with some previous research. For instance, Ashour et al. (2020) reported that while dietary coffee grounds had no effect on carcass, dressing, or giblet percentages, different levels of green coffee powder significantly enhanced liver, abdominal fat, intestinal length, and lymphoid organ percentages (spleen and Bursa of Fabricius). Notably, supplementation with 1.25 and 2.50 g/kg of green coffee powder significantly increased ($P < 0.05$) liver percentage.

Table 2
Effects of coffee ground supplementation in the diet on carcass traits of 15-week-old Japanese quails

Carcass Traits	Experimental Groups			SEM	P-value
	T ₁	T ₂	T ₃		
Live Body Weight (g)	160.67 ^b	171.95 ^a	170.09 ^a	0.66	0.0013
Dressing Weight	140.49 ^b	152.84 ^a	150.29 ^a	0.64	0.0005
Eviscerated Weight (g) ¹	129.38 ^b	139.18 ^a	138.38 ^a	0.54	0.0005
Carcass Percentage (%) ²	87.44 ^b	88.87 ^a	88.34 ^{ab}	0.11	0.0256
Gizzard (%) ³	1.23	1.31	1.24	0.01	0.3454
Heart (%)	0.98	0.95	1.03	0.01	0.3679
Liver (%)	1.43	1.45	1.44	0.01	0.9543
Spleen (%)	0.08 ^b	0.12 ^a	0.11 ^a	0.003	0.0077
Head and Neck Weight ³ (%)	10.59	11.05	11.17	0.07	0.1908
Breast and Skin Weight ³ (%)	25.94 ^b	28.38 ^a	28.64 ^a	0.09	<0.0001
Thigh Weight ³ (%)	10.55 ^b	12.03 ^a	11.58 ^a	0.07	0.0003
Drumstick Weight ³ (%)	7.49 ^b	8.28 ^a	8.85 ^a	0.07	0.0003
Wing Weight ³ (%)	6.24	6.70	6.68	0.05	0.0838
Shank Weight ³ (%)	2.14	2.23	2.26	0.03	0.5009
Abdominal Fat Weight ³ (%)	1.16	1.06	0.94	0.05	0.6527
Skeleton Weight ³ (%)	20.98	21.34	21.10	0.09	0.7160
Other Organs:					
Testis (%)	2.83 ^b	3.85 ^a	4.02 ^a	0.06	0.0002

Note. T1: Control group (commercial feed without coffee grounds), T2: Commercial feed supplemented with 10 g/kg of coffee grounds, and T3: Commercial feed supplemented with 20 g/kg of coffee grounds

^{a,b} Means with different letters in the same row show significant differences (P<0.05)

¹SEM: Standard error of the mean

Live body weight refers to the weight of broiler chickens after a 12-hour fasting period

¹Carcass weight refers to the weight of the chicken after the removal of internal organs

²Calculate as a percentage of live body weight

³Calculate as a percentage of carcass weight

Green coffee powder at 2.50 g/kg reduced abdominal fat, likely due to antioxidants decreasing malic enzyme activity, thus depressing abdominal fat deposition.

Furthermore, 1.25 and 2.50 g/kg of green coffee powder resulted in a higher percentage of spleen, and 1.25 g/kg produced a better percentage of Bursa of Fabricius and meat quality for broilers. Likewise, Hosseini-Vashan et al. (2023) confirmed that the addition of 0.9% green coffee powder to broiler diets significantly (P<0.05) improved the carcass traits such as breast, thigh and Bursa of Fabricius. Additionally, the tested levels of green coffee powder, 0.3%, 0.6%, 0.9%, 1.2%, and 1.5%, significantly decreased abdominal fat in broiler chickens compared to the control group. In line with these findings, crude coffee extract was found to significantly (P<0.05) contribute to the increase in carcass percentage,

breast meat, thigh and fillet of broiler chickens, but reduced the abdominal fat compared to control chick (Ghanima et al., 2021). The bioactive compounds present in coffee are responsible for these improvements in meat quality, indicating that the use of coffee extract as a feed additive in poultry production can improve the quality of meat produced. There has been further support for the beneficial effects of coffee-derived compounds on the productive performance of poultry, by Yachai et al. (2022), who found that feeding Thai native chickens with 2.0 g/kg coffee silver skin significantly ($P < 0.05$) enhanced both productive performance and antioxidant content in the breast muscle, as well as improving the morphology of the intestines.

Conversely, other studies like Klompanya et al. (2024) found no significant difference in broiler carcass percentage, breast meat, and thigh weights when the spent coffee grounds were added at 5% and 10% concentration. On the other hand, 5% and 10% of coffee grounds significantly affected liver weight ($P < 0.05$) and 10% significantly decreased abdominal fat and improved wing percentage, which may be attributed to the metabolism of specific ingredients of the coffee ground. The findings of the present study confirm the effectiveness of the use of coffee grounds to improve the carcass properties of the Japanese quail, which probably resulted from its rich and varied active substances. The essential compounds found in coffee are carbohydrates, minerals, caffeine, chlorogenic acid, proteins and lipids, all of which have important pharmacological and biological properties, such as protection against oxidative damage in animal cells, and are known to have antioxidant and free radical scavenging properties (Briand et al., 1996). The antioxidant activity of phenolic compounds and flavanols has also been identified by other scientists as an important property that can help to prevent damage to cells caused by free radicals (Simoh et al., 2018). In keeping with the above mechanistic explanations, Ko et al. (2012) also found that dried coffee meal had antioxidant activity in vitro, and it was a natural source of antioxidants without affecting broiler growth performance. In the small intestine and liver, glutamate thiosemicarbazone is not used as a food source by birds, and therefore, it did not affect the concentrations of antioxidant defence enzymes (glutathione peroxidase, superoxide dismutase, and glutathione S-transferase) or the concentration of the antioxidant defence molecule glutathione. However, in the liver, birds fed 0.5% dry coffee meal showed significantly reduced ($P < 0.05$) lipid peroxidation when compared to the control. Additionally, Vargas-Sánchez et al. (2019) found that the use of natural ingredients such as medicinal herbs, plants, vegetables and spices in Japanese quail diets can improve the quality of the carcass and meat due to their antioxidant effect.

However, this effect is dependent on the concentration, type, and conformation of the molecules present, which can influence the absorption and metabolism of active compounds, enabling or disabling their antioxidant or antibacterial functions. It is also important to consider that high levels of some natural ingredients may adversely affect

quail carcass and meat. Thus, the active ingredients in coffee grounds appear to enhance the immune system response, reduce inflammation, and promote overall animal health, thereby improving the carcass quality of Japanese quails. This study elucidated that coffee grounds can serve as a viable feed ingredient for animal production, with supplementation at 10 and 20 g/kg demonstrating an improvement in the carcass characteristics of 15-week-old Japanese quails.

Meat Quality

Proximate Composition

Coffee grounds significantly affected ($P < 0.05$) the proximate composition of Japanese quail breast meat, as shown in Table 3. Birds fed either 10 g/kg (T2) or 20 g/kg (T3) of coffee ground showed a significant increase in crude protein and dry matter content when compared with the control birds. The quantitative improvement regarding crude protein was found to be in the range of 21.36 to 22.29% in the T3 group, which was 4.35% higher than in the control group. Similarly, dry matter increased from 24.03% to 24.82% in Treatment T3, which resulted in an increase in nutrient density by 3.29%. At the same time, crude fat content in the breast meat was significantly lowered in the presence of the inclusion of coffee grounds (from 2.23% in the control group to 2.11% in T2 and 2.03% in T3). The results of the study are that the meat quality of quails was improved by the addition of the active ingredient contained in coffee grounds, which led to an increase in crude protein and crude dry matter percentages and a decrease in crude fat percentage in the breast meat.

The present investigation revealed that the crude protein, crude fat, ash and moisture content in coffee grounds were 14.20%, 3.50%, 2.50% and 8.0%, respectively. These results are consistent with the nutritional content of coffee grounds. Arabica spent coffee grounds are rich in various nutritional components such as nitrogen-free extract, protein, fat, fibre, ash, and gross energy, which were reported by Klompanya et al. (2024) as 48%, 12.86%, 3.70%, 17.34%, 1.36%, and 5,184.4 kcal/kg, respectively. Moreover, they reported levels of calcium (0.18%) and phosphorus (0.05%). Likewise, Kourmentza et al. 2018 reported that coffee grounds are 13.5% protein, 2.3% fat and 1.3% ash. The increased proximate composition of the breast meat in Japanese quails may be partly due to these active nutritional constituents present in the grounds of coffee.

Meat Quality

pH and Drip Loss Values

The effects of coffee ground supplementation in the diet on the meat quality of the breast meat of Japanese quail at 15 weeks of age are presented in Tables 4 and 5. The results indicated that adding the diet with coffee grounds at 10 g/kg (T2) and 20 g/kg (T3) did not result in statistically significant changes to the pH levels or water-holding capacity

(drip loss value) of the breast meat. Immediately after slaughter (pH_0), the values remained stable across groups, ranging from 6.21 to 6.26. After 24 hours (pH_{24}), pH values slightly decreased to a range of 6.09 to 6.12 but remained consistent between the control and supplemented groups. Similarly, drip loss—a key indicator of meat moisture retention—showed no significant difference, with the control group at 3.15% compared to 3.03% in the T3 group, representing a negligible reduction of approximately 3.8%.

Table 3

Effects of coffee ground supplementation in the diet on proximate composition in breast meat of Japanese quail at 15 weeks of age

Experimental groups	Proximate Composition in Breast Meat (%)		
	Crude Protein	Crude Fat	Dry Matter
T1	21.36 ^b	2.23 ^a	24.03 ^b
T2	22.16 ^a	2.11 ^b	24.75 ^a
T3	22.29 ^a	2.03 ^b	24.82 ^a
SEM	0.04	0.01	0.04
P-value	<0.0001	0.0004	0.0007

Note. T1: Control group (commercial feed without coffee grounds), T2: Commercial feed supplemented with 10 g/kg of coffee grounds, and T3: Commercial feed supplemented with 20 g/kg of coffee grounds

^{a,b} In the same column, means with different letters differ significantly ($P < 0.05$)

¹SEM: Standard error of the mean

Table 4

Effects of coffee ground supplementation in diet on pH and colour values of Japanese quail breast meat at temperature 0-4 °C for 0 hours (pH_0), within 45 minutes after slaughter

Item	Experimental Groups			SEM	P-value
	T1	T2	T3		
pH Value (pH_0) of Breast Meat	6.26	6.21	6.24	0.01	0.5149
Colour Value of Breast Meat:					
Lightness (L^*)	44.24	44.34	44.50	0.08	0.7567
Redness (a^*)	11.50	12.21	12.14	0.09	0.2024
Yellowness (b^*)	10.34 ^b	10.85 ^{ab}	10.96 ^a	0.06	0.0464
Colour Value of Skin:					
Lightness (L^*)	45.35	45.39	45.34	0.07	0.9844
Redness (a^*)	10.32	10.52	10.82	0.07	0.3172
Yellowness (b^*)	11.05 ^b	11.87 ^a	11.98 ^a	0.08	0.0221

Note. T1: Control group (commercial feed without coffee grounds), T2: Commercial feed supplemented with 10 g/kg of coffee grounds, and T3: Commercial feed supplemented with 20 g/kg of coffee grounds

^{a,b} Means with different letters in the same row show significant differences ($P < 0.05$)

¹SEM: Standard error of the mean

pH_0 is the pH value within 45 minutes after slaughter

Table 5

Effects of coffee ground supplementation in diet on pH, colour, and drip loss values of Japanese quail breast meat at temperature 0-4 °C for 24 hours (pH₂₄) after slaughter

Item	Experimental Groups				
	T1	T2	T3	SEM	P-value
pH Value (pH ₂₄) of Breast Meat	6.09	6.10	6.12	0.01	0.7104
Colour Value of Breast Meat:					
Lightness (<i>L</i> *)	45.34	34.69	45.87	0.07	0.2833
Redness (<i>a</i> *)	12.51	12.83	12.73	0.06	0.5219
Yellowness (<i>b</i> *)	11.79 ^b	12.64 ^a	12.81 ^a	0.08	0.0184
Colour Value of Skin:					
Lightness (<i>L</i> *)	46.60	46.86	46.76	0.07	0.6992
Redness (<i>a</i> *)	11.57	11.90	11.75	0.06	0.4784
Yellowness (<i>b</i> *)	12.10 ^b	12.84 ^a	12.99 ^a	0.05	0.0012
Drip loss (%)	3.15	3.05	3.03	0.01	0.2114

Note: T1: Control group (commercial feed without coffee grounds), T2: Commercial feed supplemented with 10 g/kg of coffee grounds, and T3: Commercial feed supplemented with 20 g/kg of coffee grounds

^{a,b} Means with different letters in the same row show significant differences (P<0.05)

¹SEM: Standard error of the mean

pH₂₄ is the pH value measured 24 hours after slaughter

Colour Value

Regarding the colour of breast meat, the current study found that the lightness (*L**) and redness (*a**) of the breast meat were unaffected by the diet; there was a significant quantitative increase in the yellowness (*b**). Within 45 minutes of slaughter, the yellowness value increased from 10.34 in the control (T1) to 10.96 in the T3 group, an improvement of roughly 6%. This trend became more pronounced 24 hours post-slaughter; the T2 (12.64) and T3 (12.81) groups showed significantly higher yellowness compared to the control (11.79). Specifically, the 20 g/kg supplementation (T3) resulted in an 8.6% increase in breast meat yellowness compared to the control group.

For the skin colour of breast meat, the most notable statistical improvements occurred in the colouration of the quail's skin, particularly regarding yellowness. In the initial 45-minute post-slaughter, skin yellowness rose from 11.05 (T1) to 11.98 (T3), a significant increase of approximately 8.4%. At 24 hours post-slaughter, this difference remained significant, with the T3 group reaching a value of 12.99 compared to the control group (12.10). This represents a 7.3% enhancement in skin yellowness for the T3 group. Additionally, the treatment had no significant impact on the lightness (*L**) or redness (*a**) of the meat or skin at any recorded interval, suggesting that coffee grounds specifically influence the yellow pigment profile without darkening the product.

Warriss (2000) reported typical chicken meat pH values of 6.4-7.0 at 0 hours and 5.9-6.0 at 24 hours post-slaughter. Other research indicates that Japanese quail breast meat pH values 24 hours post-slaughter range from 6.1 to 6.3, with breast meat generally having slightly lower values (Genchev et al., 2008). The ultimate pH of meat is primarily determined by muscle glycogen levels, which are significantly influenced by pre-slaughter stressors and locomotor activity (Genchev et al., 2008). As Sanchai (2000) explained, upon an animal's death, oxygen, glucose, and free fatty acid transport to muscles ceases, disrupting oxygen-dependent ATP production. The body then resorts to anaerobic glycolysis, breaking down stored glycogen to produce ATP and lactic acid, which subsequently lowers meat pH from an initial neutral level to a final range of 5.3-5.7 within 24 hours as glycogen is depleted. Genchev et al. (2008) also noted that Japanese quail meat is rich in essential amino acids, with breast meat typically containing 22.23-23.38% protein and 2.21-2.75% fat, and pectoral muscles having 21.68-22.39% free water. The various active ingredients in coffee grounds are responsible for the increase in the yellowness of breast meat and skin in Japanese quails fed coffee grounds. These results are somewhat contradicted by the study by Ashour et al. (2020), who reported that the supplementation of green coffee powder at 2.5 g/kg of broiler feed resulted in a significant increase in the lightness (L^*) and redness (a^*) of the broiler breast meat, which could be attributed to the reduction of fat and colour oxidation. It is well known that compounds obtained from coffee have beneficial effects. Coffee is also high in chlorogenic acid compounds and has been reported as having significant antioxidant and anti-inflammatory properties and other beneficial effects (Liang & Kitts, 2016). Coffee extract has been shown to possess high in vitro antioxidant activity in previous studies, making it a useful natural source of antioxidants, without negative effects on broiler growth performance. In addition, the relative weight of the thymus was significantly higher ($P < 0.05$), and the blood albumin level was raised in birds fed 0.5% and 1.0% of dried coffee meal, while there was no significant difference in other blood constituents (glucose, triglycerides, and cholesterol) (Ko et al., 2012). Coffee meal (0.5%) did not affect the antioxidant defence system, such as the activities of superoxide dismutase, hepatic glutathione peroxidase, and glutathione S-transferase; however, in birds, there was a significant ($P < 0.05$) decrease in hepatic lipid peroxidation with the addition of 0.5% coffee meal (Ko et al., 2012). The potential and benefits of natural plant extracts with antioxidant and antibacterial properties to preserve meat products (Tandale et al., 2024). Consistent with this, another researcher confirmed that coffee grounds could increase feed efficiency, increase nutrient digestion, promote antioxidant activity, and support immune response due to the presence of total phenolic compounds, such as chlorogenic acid, protocatechuic acid, and flavonoids, all known for their high antioxidant activity (Solange et al., 2011). These compounds likely contribute to the observed improvements in the physical characteristics of Japanese quail breast meat.

Faecal Pathogenic Bacteria

The study investigated the effects of coffee ground supplementation in the diet on faecal pathogenic bacteria of 15-week-old Japanese quails. The results found that supplementing Japanese quail diets with coffee grounds significantly reduced the populations of *E. coli* and *Salmonella* spp. in their faeces, as shown in Table 6. In the control group (T1), *E. coli* levels were measured at 7.6181 log₁₀ CFU/g. When quails were fed a diet supplemented with 10 g/kg of coffee grounds (T2), the *E. coli* population dropped to 7.4815 log₁₀ CFU/g, representing a decrease of approximately 1.79%. Increasing the supplementation to 20 g/kg (T3) resulted in a slightly further reduction to 7.4668 log₁₀ CFU/g, a 1.99% improvement over the control. These reductions were statistically significant (P<0.0001).

The same trend was observed for *Salmonella* spp., which also showed a downward trend, indicating the antibacterial effects of the coffee ground additive. The log₁₀ CFU/g of the control group was 7.2392. The addition of 10 g/kg (T2) to the feed lowered this to 7.1353 log₁₀ CFU/g, which is a quantitative improvement of 1.44%. The higher dose of 20 g/kg (T3) was even more effective, resulting in 7.0708 log₁₀ CFU/g (a 2.33% reduction from the control group). From the current study, it was concluded that coffee ground supplementation is a highly consistent method for reducing the population of enteric pathogens in 15-week old Japanese quails.

This study confirmed previous research, which showed the antimicrobial activity of coffee by-products. The use of 2.5 g/kg of green coffee powder significantly reduced the levels of *Salmonella* spp., *E. coli*, yeasts and moulds, and *Enterococcus* spp., and it also increased the levels of the beneficial lactic acid bacteria (Ashour et al., 2020). Similarly, Yachai et al. (2022) reported that the cecum of Thai native chickens had a significant reduction in the populations of *Salmonella* sp. and *E. coli* with 2.0g/kg of coffee silver skin (P<0.05).

Table 6
Effects of coffee ground supplementation in the diet on *E. coli* and *Salmonella* spp. populations in the faeces of 15-week-old Japanese quails

Experimental groups	<i>E. coli</i> (log10 CFU/g)	<i>Salmonella</i> spp. (log10 CFU/g)
T1	7.6181 ^a	7.2392 ^a
T2	7.4815 ^b	7.1353 ^b
T3	7.4668 ^b	7.0708 ^b
SEM	0.006	0.007
P-value	<0.0001	<0.0001

Note. T1: Control group (commercial feed without coffee grounds), T2: Commercial feed supplemented with 10 g/kg of coffee grounds, and T3: Commercial feed supplemented with 20 g/kg of coffee grounds
^{a,b} Means with different letters in the same column show significant differences (P<0.01)
¹SEM: Standard error of the mean

Additionally, Ashour et al. (2020) reported that the extract of green coffee inhibited the growth of several bacterial strains with MICs of 100, 80, 80, 60, 60 and 50 µg/mL in the case of *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC BAA-1705, *Escherichia coli* ATCC 43886, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579 and *Bacillus subtilis* ATCC 23857, respectively, while MBCs were 150, 120, 120, 100, 100 and 80 µg/mL, respectively. This could be attributed to the presence of different bioactive compounds of the coffee grounds that had high inhibitory potential on pathogenic bacteria growth. The antibacterial activity of the coffee grounds that was observed is probably due to the abundance of bioactive compounds in the coffee grounds. The coffee grounds are rich in total phenolics (55.4 ± 1.11 mg GAE g⁻¹ extract) and flavonoids (14.2 ± 0.42 mg QE g⁻¹ extract), which are all effective against microorganisms.

Additionally, coffee grounds are also rich in triglycerides, fatty acids, and antioxidant compounds. These fatty acids exhibit a wide range of biological activities, including anti-inflammatory, anticancer, antibacterial, antidiabetic, and anti-atherosclerotic effects (Al-Asmari et al., 2020). However, the mechanisms through which these phytochemicals act within the gastrointestinal tract remain unclear and require further investigation to identify the specific active compounds and their metabolites. Similarly, Sharma et al. (2022) reported that herbal compounds show strong therapeutic potential against infectious pathogens, particularly bacteria, emphasising the need for additional studies on the diverse phytochemical constituents of plants, their mechanisms of action, and possible synergistic interactions responsible for their antibacterial properties. The consistent reduction of pathogenic bacteria reported in this study demonstrated that coffee grounds could serve as a natural and sustainable feed ingredient for controlling microbial populations in livestock, providing a promising alternative to conventional antibiotics and harmful chemicals used in animal production.

CONCLUSION

Dietary supplementation of coffee grounds at levels of 10 and 20 g/kg in Japanese quails significantly enhanced live body weight, carcass yield, and the relative proportions of valuable commercial cuts, including the breast, thigh, and drumstick. In addition, positive effects were observed on spleen and testes weights. Coffee ground supplementation also improved breast meat quality by increasing dry matter, crude protein content, and meat yellowness while concurrently reducing faecal populations of *E. coli* and *Salmonella* spp.

These findings indicated that coffee grounds can be effectively incorporated into commercial feed formulations as a cost-efficient and bioactive agro-industrial byproduct to enhance growth performance and meat quality. Furthermore, their antimicrobial properties suggest that coffee grounds may serve as a natural alternative for improving gut health in poultry production. Nevertheless, further studies are needed to determine optimal

supplementation levels, elucidate the underlying physiological mechanisms, and assess the long-term effects on animal health and environmental sustainability.

CONFLICTS OF INTEREST

This research was conducted independently for non-profit purposes, and the author declares no conflicts of interest.

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REFERENCES

- Association of Official Analytical Chemists (AOAC). (2000). *Official methods of analysis* (17th ed.)
- Al-Asmari, K. M., Abu Zeid, I. M., & Al-Attar, A. M. (2020). Medicinal properties of Arabica coffee (*Coffea arabica*) oil: An overview. *Advancements in Life Sciences*, 8(1), 20-29. <https://doi.org/10.62940/als.v8i1.1024>
- Ashour, E. A., Abd El-Hack, M. E., Shafi, M. E., Alghamdi, W. Y., Taha, A. E., Swelum, A. A., Tufarelli, V., Mulla, Z. S., El-Ghareeb, W. R., & El-Saadony, M. T. (2020). Impacts of green coffee powder supplementation on growth performance, carcass characteristics, blood indices, meat quality and gut microbial load in broilers. *Agriculture*, 10(10), Article 457. <https://doi.org/10.3390/agriculture10100457>
- Batool, F., Bilal, R. M., Hassan, F. U., Nasir, T. A., Rafeeque, M., Elnesr, S., Farag, M. R., Mahgoup, H. A. M., Naiel, M. A. E., & Alagawany, M. (2021). An updated review on behaviour of domestic quail with reference to the negative effect of heat stress. *Animal Biotechnology*, 34(2), 424-437. <https://doi.org/10.1080/10495398.2021.1951281>
- Bertrand, B., Boulanger, R., Dussert, S., Ribeyre, F., Berthiot, L., Descroix, F., & Joët, T. (2012). Climatic factors directly impact the volatile organic compound fingerprint in green Arabica coffee bean as well as coffee beverage quality. *Food Chemistry*, 135(4), 2575-2583. <https://doi.org/10.1016/j.foodchem.2012.06.060>
- Castanon, J. I. R. (2007). History of the use of antibiotics as growth promoters in European poultry feeds. *Poultry Science*, 86(11), 2466-2471. <https://doi.org/10.3382/ps.2007-00249>
- DaMatta, F. M., Ronchi, C. P., Maestri, M., & Barros, R. S. (2007). Ecophysiology of coffee growth and production. *Brazilian Journal of Plant Physiology*, 19(4), 485-510. <https://doi.org/10.1590/S1677-04202007000400014>
- Genchev, A., Mihaylova, G., Ribarski, S., Pavlov, A., & Kabakchiev, M. (2008). Meat quality and composition in Japanese quails. *Trakia Journal of Sciences*, 6(4), 72-82. http://www.uni-sz.bg/tsj/TJS-Vol.6%20N4%202008/Genchev_kachestvoEn.pdf
- Ghanima, M., Shukry, M., El-Sawy, H., & Ibrahim, A. (2021). Effect of green coffee, green tea, cinnamon and rosemary extracts on productive performance, feeding behaviour, immunity and oxidative stress

- in broilers suffering heat stress. *Alexandria Journal of Veterinary Sciences*, 69(1), 52-60. <https://doi.org/10.5455/ajvs.59855>
- Ho, L., Varghese, M., Wang, J., Zhao, W., Chen, F., & Knable, L. A. (2012). Dietary supplementation with decaffeinated green coffee improves diet-induced insulin resistance and brain energy metabolism in mice. *Nutritional Neuroscience*, 15(1), 37-45. <https://doi.org/10.1179/1476830511Y.0000000027>
- Hosseini-Vashan, S. J., Manochchri, D., Afzali, N., & Yonesi, H. (2023). *Green coffee powder: Effect on growth performance, biochemical blood indices, and intestine morphology in broiler chickens* [Preprint]. Research Square. <https://doi.org/10.21203/rs.3.rs-2521455/v1>
- Ismail, L., Anuar, M. S., & Shamsudin, R. (2014). Physical properties of Liberica coffee (*Coffea liberica*) berries and beans. *Pertanika Journal of Science and Technology*, 22(1), 65-79. <https://doi.org/10.47836/pjtas.47.2.13>
- Klompanya, A., Mitchaothai, J., & Thammakarn, C. (2024). Effects of partial substitution of feed with spent coffee grounds on growth performance and carcass quality in broilers. *International Journal of Agricultural Technology*, 20(2), 575-586. <http://www.ijat-aatsea.com>
- Ko, Y. H., Kang, S. Y., & Jang, I. S. (2012). Effects of dietary supplementation of coffee meal on growth performance, blood biochemical profiles and antioxidant defense system in broiler chickens. *Korean Journal of Poultry Science*, 39(3), 223-232. <https://doi.org/10.5536/KJPS.2012.39.3.223>
- Kourmentza, C., Economou, C. N., Tsafrakidou, P., & Kornaros, M. (2018). Spent coffee grounds make much more than waste: Exploring recent advances and future exploitation strategies for the valorisation of an emerging food waste stream. *Journal of Cleaner Production*, 172, 980-992. <https://doi.org/10.1016/j.jclepro.2017.10.088>
- Liang, N., & Kitts, D. D. (2016). Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients*, 8(1), Article 16. <https://doi.org/10.3390/nu8010016>
- Marcel, B. K. G., André, K. B., Viviane, Z.-T., & Séraphin, K.-C. (2011). Potential food waste and by-products of coffee in animal feed. *Electronic Journal of Biology*, 7(4), 74-80.
- Moreng, R. E., & Avens, J. S. (1985). *Poultry science and production*. Reston Publishing Company.
- Murwani, R., Anggraeni, R., Muslih, M. H., Yogantara, A. O., & Mulyono. (2024). Egg production, blood profile, and histopathology in Japanese quail with phytogetic additives. *Tropical Animal Science Journal*, 47(3), 321-332. <https://doi.org/10.5398/tasj.2024.47.3.321>
- Nillian, E., Ismail, N. S., Boli, M. E., Buyong, N. L., Sng, N. N., Adeni, D. S. A., & Hussini, A. A. S. A. (2020). The feasibility study of physicochemical properties of Sarawak Liberica sp. coffee pulp. *Pertanika Journal of Tropical Agricultural Science*, 43(4), 477-490. <https://doi.org/10.47836/pjtas.43.4.05>
- Phromnoi, S., Chumngoen, W., Puangmalee, T., & Nuchchanart, W. (2025). Carcass traits, physicochemical characteristics, fatty acid, and protein profile of Khiew Phalee, Pradu Hang Dam and broiler chicken meat. *Tropical Animal Science Journal*, 48(1), 57-67. <https://doi.org/10.5398/tasj.2025.48.1.57>
- Posan, P., Suler, A., Nistor, L., Hodosan, C., Marius, M., & Udriou, A. (2023). Use of medicinal plants with galactogenic effect as a food supplement to increase milk production in dairy animals: A review. *Indian Journal of Animal Research*, 57(6), 677-683. <https://doi.org/10.18805/IJAR.BF-1527>

- Sanchai, J. (2000). *Meat science and technology*. Thanabaan Interprint Company Limited.
- SAS Institute. (2017). *SAS/STAT 14.3 user's guide*. SAS Institute Inc.
- Sharma, N., Mishra, A. K., Kumar, A., Srivastava, A., Gururaj, K., Singh, D. D., Singh, T. P., & Pawaiya, R. V. S. (2022). Antibacterial activity of traditionally used plants against the resistant enteropathogenic *Escherichia coli*. *Indian Journal of Animal Research*, 56(6), 759-763. <https://doi.org/10.18805/IJAR.B-4844>
- Simoh, S., Shin, S. Y., Rahim, F. A., Ahmad, M. A., & Zainal, A. (2018). Comparative analysis of metabolites and antioxidant potentials from different plant parts of *Curcuma aeruginosa* Roxb. *Sains Malaysiana*, 47(12), 3031-3041. <https://doi.org/10.17576/jsm-2018-4712-13>
- Solange, I. M., Ballesteros, L. F., Martins, S., & Teixeira, J. A. (2011). Extraction of antioxidant phenolic compounds from spent coffee grounds. *Separation and Purification Technology*, 83, 173-179. <https://doi.org/10.1016/j.seppur.2011.09.036>
- Tajik, N., Tajik, M., Mack, I., & Enck, P. (2017). The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: A comprehensive review of the literature. *European Journal of Nutrition*, 56(7), 2515-2545. <https://doi.org/10.1007/s00394-017-1379-1>
- Tandale, A. T., Nayak, B. B., Xavier, K. A. M., Devangre, A. A., Gore, S. B., & Balange, A. K. (2024). Phenolic compounds and bioactive properties of black tea powder. *Indian Journal of Animal Research*, 58(2), 342-346. <https://doi.org/10.18805/IJAR.B-4849>
- United States Food and Drug Administration (FDA). (2020). *Bacteriological analytical manual (BAM)*. <https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam>
- Vali, N. (2008). The Japanese quail: A review. *International Journal of Poultry Science*, 7(9), 925-931. <https://doi.org/10.3923/ijps.2008.925.931>
- Vargas-Sánchez, R. D., Ibarra-Arias, F. J., Torres-Martínez, B. M., Escalante, A. S., & Torrescano-Urrutia, G. R. (2019). Use of natural ingredients in Japanese quail diet and their effect on carcass and meat quality - A review. *Asian-Australasian Journal of Animal Sciences*, 32(11), 1641-1656. <https://doi.org/10.5713/ajas.18.0800>
- Warriss, P. D. (2000). *Meat science: An introductory text*. CABI Publishing. <https://doi.org/10.1079/9780851994246.0000>
- Wattanachant, S. (2003). *Chemical composition, properties and structure of muscle affecting textural characteristics of meat from Thai indigenous and broiler* [Doctoral dissertation, Prince of Songkla University]. Prince of Songkla University Knowledge Repository.
- Wattanachant, S., Benjakul, S., & Ledward, D. A. (2004). Composition, colour and texture of Thai indigenous and broiler chicken muscles. *Poultry Science*, 83(1), 123-128. <https://doi.org/10.1093/ps/83.1.123>
- Yachai, M., Srinual, O., Khamtavee, P., Punyatong, M., Lumsangkul, C., Pintalerd, N., & Tapingkae, W. (2022). Effects of coffee silver skin supplementation in Thai native crossbred chicken (Pradu-Hangdum) diets on growth performance, antioxidant status in breast meat, microbial population in cecum, intestinal morphology, and gene expression. *King Mongkut's Agricultural Journal*, 40(3), 286-301.

The Effectiveness of Response Surface Methodology (RSM) as a Reliable Tool for Performance Optimisation in Azo Dye Removal Using Cellulose Nanofibril (CNF) Filter Paper

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ABSTRACT

The potential of *Neolamarckia cadamba* cellulose nanofibril (CNF) filter paper as an azo dye treatment in a crossflow filtration system was investigated. The water permeability analysis was performed to assess the suitability of the fabricated CNF filter paper for filtration. Three operating parameters, cellulose dosage ratio, initial pH and initial concentration of methyl orange (MO) dye were optimised by central composite design (CCD) and response surface methodology (RSM). The results indicated that the model successfully predicted the relationship between the CNF filter paper and MO dye, demonstrating a high colour removal efficiency of 97.28% and an optimum normalised flux of 0.9748. These values were obtained under optimal conditions of a 50:50 cellulose ratio, pH 4, and an initial MO concentration of 600 mg/L. Fouling analysis using the Wiesner and Aptel model suggested that cake layer formation occurred in two stages under the optimum conditions. Overall, the findings indicate that RSM is useful for identifying suitable operating conditions, and the CNF filter paper shows good potential as a filtration material for azo dye removal.

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INTRODUCTION

With the various environmental concerns, the growing amount of contaminated wastewater released into aquatic systems in textile and related industries, with the use of synthetic dyes, has become a major concern. Industries such as textiles, paper, leather, and cosmetics contribute significantly to dye pollution because of the extensive use of colouring agents during manufacturing processes (Javaid et al., 2021). Most synthetic dyes are difficult to degrade owing to their stable chemical structures and resistance to biological decomposition. Their resistance to degradation allows the dyes to remain in the environment for long periods. Previous studies have reported that exposure to certain synthetic dyes may cause adverse effects on human health, including skin and eye irritation, liver damage, and potential mutagenic or carcinogenic effects (Singh et al., 2021; Thiruppathi et al., 2021).

Various methods have been explored for the removal of dyes from wastewater, such as adsorption (Hanafy, 2021; Hussain et al., 2021; Lin et al., 2021a), oxidation processes (Pu et al., 2022; Türgay et al., 2011), electrochemical treatment (Petcu et al., 2016), and nanofiltration membrane systems (Amin & Nizam, 2016; Aryanti et al., 2018; Bai et al., 2023; Miao et al., 2021). Cellulose nanofibril (CNF) nanopapers have been the focus of study in recent years for use as a membrane because of the excellent adsorption properties and the possibility of using them for filters in wastewater treatment. Although these benefits, their usage is still restricted due to the low water permeability based on the dense fibre structure. Hence, efforts towards the development of nanopaper need to be directed towards the creation of a porous network of cellulose with an appropriate pore size to enhance the water transport performance (Mautner et al., 2019). Porosity-enhancing cellulose-based membranes were reported to render good mechanical stability along with a high rejection performance by Gu et al. (2023).

CNF has been manufactured from various sources of raw materials, such as wood-based materials, non-wood fibres and agricultural wastes. *Neolamarckia cadamba* or locally known as *Kelempayan*, is one of the possible materials, commonly available in South China and South Asian countries. The tropical wood species is a high cellulose content wood species, which is suitable to be used for the production of bleached pulp and then used for the production of CNF (Latifah et al., 2020). The tree species used in this study was *Neolamarckia cadamba* due to its availability from forest plantations in Malaysia and its potential for developing value-added CNF filter paper material.

In this study, nanofiltration membrane technology incorporated into a crossflow filtration system was investigated as an alternative approach due to its capability to provide more consistent and sustainable filtration performance. Cross-flow filtration systems are typically continuous filtering systems adapted from hybrid membrane photocatalytic reactors (MPR). The permeation of the treated solution influences the efficiency and capabilities of this system (Desa et al., 2019; Hairom et al., 2015; Sidik et al., 2019).

Compared with traditional filtration methods, the crossflow filtration system employs continuous tangential flow to reduce pollutant transport into the treated effluent.

Design of Experiments (DOE) is a group of strategies and processes used primarily to investigate data referring to specific variables of a particular subject of study (El-Sheekh et al., 2023). To maximise the filtration of textile wastewater utilising CNF filter paper, the experimental factorial design of the central composite design (CCD) was implemented in this study. Meanwhile, response surface methodology (RSM) is an empirical approach based on the DOE principle, which is used in the design, modelling, and optimisation of experimental factors and their interactions with responses, compared to the one-factor-at-a-time technique (K et al., 2021; Yadav et al., 2021; Veza et al., 2023). The application of RSM has evolved from simple optimisation to complex fouling analysis, as demonstrated by Chen et al. (2024), who utilised RSM to decode the interactions between operational variables and membrane life-cycles. This study is an extension of these developments using a fine-tuned CCD-RSM for processing *Neolamarckia cadamba*, the unique feedstock.

Membrane fouling behaviour was analysed using the Wiesner and Aptel model, which was adapted from Hermia's classical blocking filtration laws (Hermia, 1985; Wiesner & Aptel, 1996). The modelling approach assumed constant membrane resistance, consistent pressure drops, and stable specific cake resistance throughout the filtration process (Desa et al., 2019). In addition, the experimental data enabled further interpretation of the flux decline behaviour during the filtration process.

Although cellulose-based nanopapers have been widely investigated for dye adsorption and dead-end filtration applications, studies focusing on the optimisation of methyl orange (MO) removal using enzymatically pre-treated *Neolamarckia cadamba* CNF filter paper in a continuous crossflow filtration system remain limited. This study addresses this gap by establishing a predictive model for both colour removal and flux decline mechanisms in a crossflow configuration. Thus, the CCD model designed based on RSM was implemented to assess the performance of the CNF filter paper and the optimum conditions based on the parameters. Blocking mechanism analysis was further employed to investigate the flux decline behaviour and to explain the fouling progression occurring on the nanocellulose filter paper surface.

MATERIALS AND METHODS

Reagents and Materials

In this study, only analytical-grade chemicals and reagents were employed. Sulphuric acid (96%) was purchased from Fisher Scientific (UK), and fully hydrolysed polyvinyl alcohol (PVA) was obtained from Sigma Aldrich (US). Sodium hydroxide, sodium sulphide, ethanol, and toluene were purchased from R&M Chemical (UK), while glacial acetic acid was acquired from Ajax Finechem (Australia) and sodium chlorite from Merck (USA). R&M Chemicals (UK) supplied the methyl orange (MO) powder.

MO Dye Solution Preparation

Distilled water was used to dissolve the pigment powder at concentrations of 100-1000 mg/L to produce dye solutions. The colour intensity of the MO dye was measured by utilising a Hitachi U-3900H UV-Vis Spectrophotometer. Hydrochloric acid (HCl) and sodium hydroxide (NaOH), both manufactured by R&M Chemicals (UK), were utilised for modifying the pH. The pH was determined using a microprocessor pH metre (HQ440d multi, Hach Model). No additional buffer solutions or reagents were used during the UV-Vis absorbance measurement, apart from the HCl and NaOH used for the pH adjustment of the dye solution before analysis.

Enzymatically Pre-treated *Neolamarckia cadamba* Cellulose Nanofibril Filter Paper

In accordance with previously published work by Latifah et al. (2020), cellulose nanofibrils (CNF) from *Neolamarckia cadamba* were synthesised through kraft pulping and bleaching processes. Cellulase enzyme from *Aspergillus niger* was used as an enzymatic pre-treatment to continue the process. Approximately 2300 g of bleached pulp from the previous process stage was mixed with 8.2 g of sodium acetate ($C_2H_3NaO_2$), 2.5 g of cellulase enzyme, and 2.0 L of distilled water. The enzymatic reaction procedure was carried out for 72 hours under operational conditions with a stirring rate of 80 rpm and a temperature of 28 °C. The sample was then soaked in hot water to denature the enzyme reaction.

The preparation and fabrication procedures employed in this study were adapted from the method reported by Norrahma et al. (2023). The CNF filter paper used in this study was prepared by combining cellulose nanofibrils (CNF) and bleached pulp at various mixing ratios, namely 50:50, 60:40, 70:30, 80:20, and 90:10.

Physical Characterisation of CNF Filter Paper

Physical characterisation of the CNF filter paper was carried out by evaluating its thickness, density, porosity, and fibril diameter (Mautner & Bismarck, 2021). Thickness measurements for each cellulose composition were obtained using an L&W Micrometre instrument (Lorentzen and Wettre) at ten different locations, followed by calculation of the average value. The fibril diameters were subsequently determined through 30 repeated measurements using the Fiji distribution plugin in ImageJ (Java 8).

The mass (m , g) and thickness (d , μm) of the CNF filter paper were measured to evaluate the grammage (G , g/m^2), envelope density (ρ_e), and porosity (Φ) based on established methods reported by Janesch et al. (2020). Mass measurements were obtained using an analytical balance (A&D GF-1600, US), whereas thickness was determined using an L&W Micrometre (Code 250, Lorentzen and Wettre). The theoretical porosity was subsequently calculated using the theoretical density of pure cellulose ($\rho_c = 1500 \text{ kg/m}^3$)

as reported by Mautner and Bismarck (2021). Equations 1, 2, and 3 were applied to determine the grammage, envelope density, and porosity values, respectively.

$$G(\text{gm}^{-2}) = \frac{m(\text{g})}{\pi r^2(\text{m}^2)} \quad [1]$$

$$\rho_{\text{envelope}}(\text{kgm}^{-3}) = \frac{G(\text{kgm}^{-3})}{d(\text{m})} \quad [2]$$

$$\Phi(\%) = \left(1 - \frac{\rho_{\text{envelope}}(\text{kgm}^{-3})}{\rho_{\text{cellulose}}(\text{kgm}^{-3})}\right) \times 100 \quad [3]$$

Water Permeability Testing

The water permeability test was carried out by exposing the CNF filter paper to continuous distilled water circulation at 3 bars pressure for 30 minutes during the wetting process to avoid recurring membrane compaction. A schematic representation of the crossflow filtration setup is shown in Figure 1, comprising a 2 L reactor tank coupled with a separation unit. The permeation study was conducted using a Sterlitech™ HP4750 (USA) crossflow filtration system with CNF filter paper providing an effective filtration area of 20.60 cm².

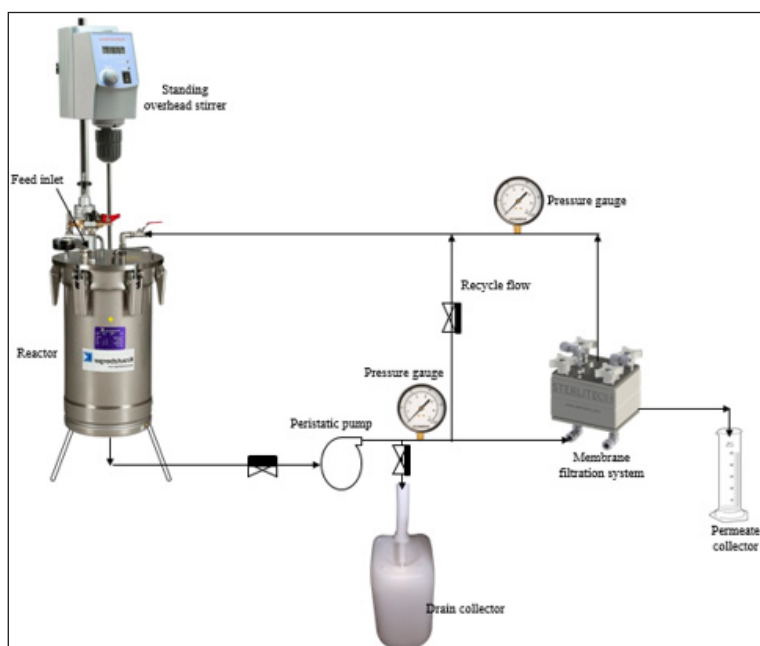


Figure 1. Schematic diagram of a crossflow filtration system

Experimental Set-up

Following the procedure established in the previously published work by Norrahma et al. (2023), various CNF filter papers with mixing ratios of CNF and bleached pulp at 50:50, 60:40, 70:30, 80:20, and 90:10 were integrated into the filtration system. The cellulose dosage was fixed at 50:50 for the RSM optimisation phase based on its superior permeability performance ($229.04 \text{ Lm}^{-2}\text{hr}^{-1}$) (Norrahma et al., 2023). During the scoping experiments, the initial pH of the MO dye solution was adjusted within the range of 3.0-10.0, whereas the dye concentration varied from 100 to 600 mg/L. Permeate flux readings were monitored every 5 minutes throughout the 3-hour filtration process. The CNF filter paper performance was then assessed under the optimal operating conditions identified in this study, namely the cellulose composition ratio, solution pH, and initial dye concentration.

Preliminary Scoping and Parameter Selection

Initial scoping experiments were first performed to establish the appropriate operating range for each experimental variable before implementing the CCD model (Hamid et al., 2016). In this study, pH (X_1) and initial MO dye concentration (X_2) were treated as independent variables, whereas colour removal (Y_1) and normalised flux (Y_2) represented the dependent responses. The range of each parameter was selected according to the findings obtained from the preliminary screening experiments.

The pH range investigated in this study was selected between 3 and 10 to represent acidic and alkaline wastewater conditions commonly applied in dye treatment studies (Amin & Nizam, 2016; Hairom et al., 2014). The dye concentration range used in this study was selected according to the ability of the CNF filter paper to remove contaminants at different concentration levels, as reported in previous studies (Hussain et al., 2021; Lin et al., 2021). Several operating conditions were fixed throughout the experiment, including the cellulose dosage ratio (50:50), operating pressure (3 bars), and filtration time (3 hours), based on the preliminary scoping tests. The applied pressure was suitable to prevent damage or rupture of the filter paper during filtration. At the same time, the filtration time selected was thought to be adequate for the system to be settled. Consequently, MO dye removal through the crossflow filtration system was evaluated at pH 3-10 and dye concentrations ranging from 100 to 600 mg/L.

Statistical Optimisation via CCD-RSM

Central composite design (CCD) in the response surface methodology (RSM) was selected in this study due to its capability to efficiently develop second-order polynomial models with a reduced number of experimental runs. Compared to conventional one-factor-at-a-time approaches, CCD allows the evaluation of interaction and quadratic effects between operational variables, making it suitable for optimisation studies involving nonlinear filtration systems.

In addition, CCD provides a reliable prediction of system performance while minimising experimental cost and time. CCD-RSM analysis was performed using Design-Expert software (Stat-Ease Inc., version 13) for experimental design, statistical modelling, response surface generation, and optimisation analysis. The relationship between the independent variables, namely pH (X_1) and initial feed concentration (X_2), and the response variables, colour removal (Y_1) and normalised flux (Y_2), was subsequently evaluated. Preliminary experiments for scoping were performed to establish the selected parameters and ranges. The optimisation study was done using pH range of 4 - 9 and initial MO dye concentration of 300 - 600 mg/L. The CCD matrix was developed from the coded level low (-1) and high (+1) of the operating conditions.

Analytical Methods

Colour concentration measurements were performed using a UV-Vis spectrophotometer (Hitachi, U-3900H) at the maximum absorption wavelength of $\lambda_{\text{max}} = 465$ nm. The degradation of the dye solution was determined from the decrease in absorbance intensity recorded at the maximum wavelength. The percentage of colour removal was then calculated using the following Equation 4.

$$\frac{C_0 - C_t}{C_0} \times 100\% \quad [4]$$

where C_0 denotes the initial colour concentration of the dye solution, whereas C_t corresponds to the remaining colour concentration after filtration at a given reaction time.

The pure water flux (J_0) of each CNF filter paper was calculated based on the permeate volume (V) passing through a unit membrane area (A) within a given filtration time (t), using the following Equation 5:

$$J_0 = \frac{V}{At} \quad [5]$$

In the periods of time t_1 and t_2 , the instantaneous permeate flow (J) at each run was determined by Equation 6:

$$J = \frac{V_2 - V_1}{A(t_2 - t_1)} \quad [6]$$

Equation 7 was applied to normalise the flux values to assess the filtration performance of the CNF filter paper under varying operational parameters. The reduction in permeate flow was further evaluated by presenting the normalised flux profile against operating time.

$$\text{Normalised flux} = \frac{\text{Solution flux } J}{\text{Pure water flux } J_0} \quad [7]$$

Membrane Fouling Mechanism

The fouling mechanism of the CNF filter paper at the optimum filtration condition was analysed based on the Wiesner and Aptel models. MATLAB R2023b was used to generate the blocking law plots and determine the corresponding fitting parameters from the filtration time data. The dominant fouling mechanism was identified using the fitted rate constant (K) and coefficient of determination (R²) values. The general equation used to analyse blocking filtration laws is presented in Equation 8, where V is the volume of permeate at time, t, and K is the coefficient depending on the flow rate and solution properties.

$$\frac{d^2t}{dV^2} = K \times \left(\frac{dt}{dV}\right)n \quad [8]$$

RESULTS AND DISCUSSION

Physical Properties and Performance of CNF Filter Paper

Figure 2 presents the envelope density and porosity behaviour of CNF filter paper as a function of thickness for different cellulose dosage compositions. Increasing the CNF proportion in the CNF-bleached pulp matrix resulted in a noticeable reduction in filter paper thickness, decreasing from $306.8 \pm 5.2 \mu\text{m}$ at the 50:50 composition to $83.9 \pm 4.5 \mu\text{m}$ at the 90:10 composition. At the same time, the envelope density increased from 195.61 to 716.51 kg/m^3 as the CNF concentration increased. Based on the theoretical density of pure cellulose (1500 kg/m^3), the porosity (Φ) ranged from 13.04% to 47.68% when the filter paper thickness decreased. This observation indicates that higher CNF content led to closer fibre arrangement, producing a denser and more compact filter paper structure.

A decrease in water permeance was observed as the cellulose dosage of the CNF filter paper increased. This may be related to the formation of a denser fibre network with fewer empty spaces, making it more difficult for water molecules to pass through the filter paper. Similar behaviour was observed from the porosity results of the fabricated CNF filter paper.

The thickness and fibril diameter of the CNF filter paper prepared with different dosage ratios of cellulose are shown in Figure 3. The thickness of the filter paper slowly decreased as the CNF content increased, while the fibril diameter increased proportionately with the CNF content. According to Mautner and Bismarck (2021), fibril network porosity is among the key factors affecting the fibril diameter within nanocellulose paper structures. The physical properties obtained for all CNF filter paper compositions are summarised in Table 1. The observed increase in porosity may be attributed to the formation of compact and densely packed nanofibrillated cellulose networks during the pressing process, which subsequently influenced the development of the fibril structure.

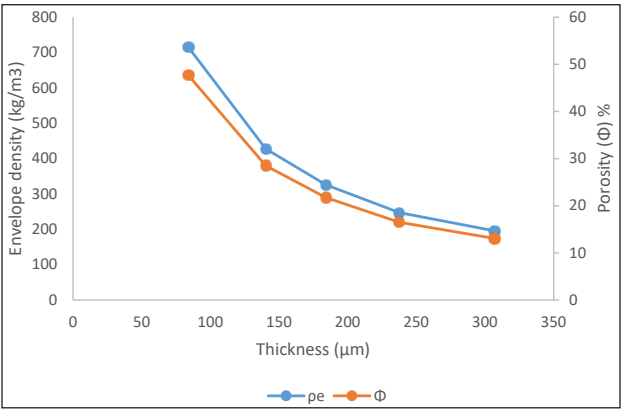


Figure 2. Envelope density and porosity evaluation based on the thickness value of the CNF filter paper

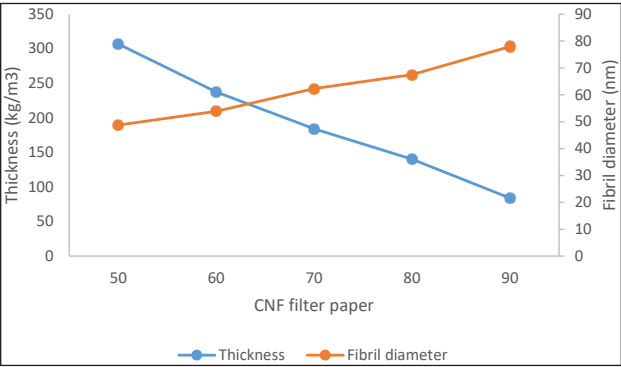


Figure 3. Effect of cellulose dosage on the thickness and fibril diameter of CNF filter paper

Table 1
Composition of *G* (grammage), thickness (*d*), envelope density (ρ_d), porosity (Φ), and fibril diameters of CNF filter paper

Mixing Ratio		G (g/m ²)	d± Std. dev. (μm)	ρ _e ±Std. dev. (kg/m ³)	Φ (%)	Fibril Diameter (nm)		
CNF	Bleached Pulp					Average	Max.	Min.
50	50	60	306.8 ± 5.2	195.61 ± 2.7	13.04	48.8 ± 18.8	105.404	19.348
60	40	60	237.6 ± 4.4	247.07 ± 3.7	16.47	53.8 ± 26.4	127.03	17.959
70	30	60	184.1 ± 11.4	326.75 ± 16.5	21.73	62.3 ± 22.3	95.817	4.277
80	20	60	140.3 ± 5.6	428.11 ± 13.9	28.51	67.5 ± 18.9	89.119	15.375
90	10	60	83.9 ± 4.5	716.51 ± 1.4	47.68	77.9 ± 51.8	171.055	16.141

Note. Std. dev. = standard deviation

Water Permeability Assessment

Maintaining stable water permeability with minimal permeance variation after membrane compaction is considered an important characteristic of high-performance membranes (Mautner & Bismarck, 2021). Despite their high rejection capability, many nanocellulose membranes remain limited by low permeability performance (Yousefi et al., 2021). Accordingly, this study evaluated the compatibility of the fabricated CNF filter paper within the crossflow filtration system under pollutant-free conditions using distilled water. The profile of distilled water flux for each CNF filter paper composition is shown in Figure 4 at different pressures of operation. Linear regression coefficient (R^2) was used to assess the consistency of membrane performance. The values of R^2 for all cellulose dosage compositions were above 0.90, which indicates good membrane performance. At 3 bars operating pressure, the permeability values for the 50:50, 60:40, 70:30, and 80:20 compositions were 229.0396, 63.7684, 8.5732, and 231.6199 $\text{Lm}^{-2}\text{h}^{-1}$, respectively. The 90:10 composition, on the other hand, ruptured the membrane at 1 bar and was not used for further analysis.

Maintaining high density alongside high porosity while yielding high permeance is a primary objective for high-quality nanopapers. This study demonstrates that the CNF filter paper is compatible with the crossflow filtration system at cellulose dosages of 50:50, 60:40, 70:30, and 80:20. The observed compatibility can be related to the formation of looser fibril networks that promoted higher porosity and greater void volume within the CNF filter paper. Despite the increased resistance caused by denser fibre packing, the addition of bleached pulp during fabrication enabled the development of larger pore structures while maintaining nanopaper density. Consequently, this contributed to the permeance behaviour and compatibility performance observed in the crossflow filtration system.

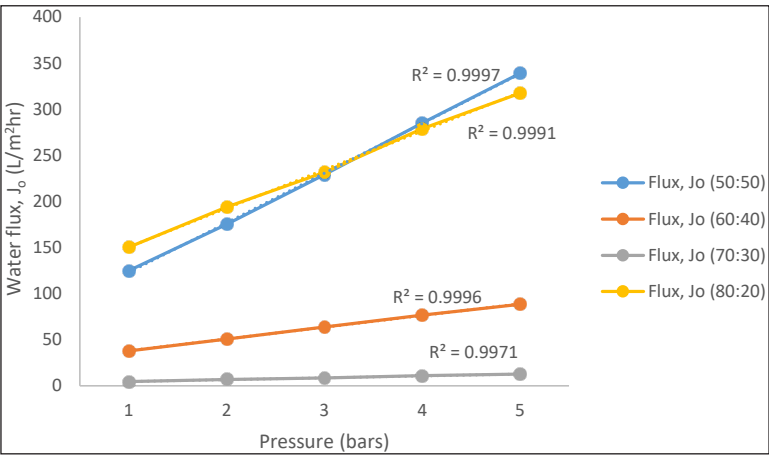


Figure 4. Distilled water flux against pressure for various cellulose dosages of CNF filter paper

CCD-RSM Optimisation for MO Dye Removal using CNF Filter Paper

CCD-RSM was employed to establish the relationship between the selected operating variables and the filtration responses. The experimental design generated 13 runs based on a 2² full factorial central composite design consisting of two independent variables: pH (X₁) and initial MO dye concentration (X₂). Colour removal (Y₁) and normalised flux (Y₂) were selected as the response variables for model evaluation. The CCD arrangement included four factorial points, four axial points, and five replicated centre points to estimate experimental uncertainty and to verify the reproducibility and suitability of the proposed model. Table 2 shows the coded factors and range and levels of independent variables, while the experimental design and corresponding results are presented in Table 3. The experimental findings demonstrated that the highest colour removal (97.28%) and normalised flux (0.9748) were achieved at the factorial design point corresponding to pH 4 and 600 mg/L initial MO dye concentration. Conversely, the centre point condition (pH 6.5 and 450 mg/L) produced the lowest performance, with colour removal and normalised flux values of 70.80% and 0.1836, respectively. The predicted responses for colour removal percentage and normalised flux were described by the second-order polynomial equation in Equation 9.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i < j} \beta_{ij} X_i X_j + \varepsilon \tag{9}$$

where Y is the measured response; x_i and x_j are the coded independent variables; β₀, β_i, β_{ii}, and β_{ij} are the regression constant, linear, quadratic, and interaction coefficients, respectively; and ε is the random error.

Table 2
Experimental ranges and levels of independent variables

Independent Variable	Factor Code	Range and Level				
		-α	-1	0	+1	+α
Initial pH	X ₁	4	2.96	6.5	10.04	9
Initial MO dye concentration (mg/L)	X ₂	300	237.87	450	662.13	600

Table 3
Experimental conditions and results of responses from the CCD

Run	Experimental Variable		Response (Colour Removal, Y ₁)		Responses (Normalised Flux, Y ₂)	
	pH, X ₁	Concentration, X ₂ (mg/L)	Experimental	Predicted	Experimental	Predicted
1	4	600	97.28	97.08	0.9748	0.9671
2	6.5	450	50.25	51.07	0.5247	0.5601
3	9	300	62.17	61.97	0.1954	0.1877
4	6.5	450	52.60	51.07	0.5695	0.5601
5	4	300	93.10	92.90	0.8745	0.8668
6	9	600	87.04	86.84	0.1589	0.1512
7	6.5	450	50.30	51.07	0.5742	0.5601
8	10	450	75.99	76.19	0.2957	0.3034
9	6.5	450	51.4	51.07	0.5484	0.5601
10	3	450	90.75	90.95	0.7251	0.7328
11	6.5	240	91.52	91.72	0.3756	0.3833
12	6.5	450	50.80	51.07	0.5836	0.5601
13	6.5	660	79.71	79.91	0.7458	0.7535

Model Fitting and Analysis of Variance

ANOVA was applied to assess the suitability of the quadratic polynomial model and to analyse the effects of the independent variables and their interactions on the response parameters (Esmi et al., 2023). The suitability of the second-order regression model was assessed using several statistical criteria, including the sequential Fisher test (F-test), lack-of-fit analysis, and other CCD adequacy parameters (Kadier et al., 2022). The statistical outcomes for each response are presented in Table 4. In general, a significant quadratic model is associated with high F-values and low probability values. The developed models in this study produced F-values of 18.05 for colour removal and 6.62 for normalised flux. Model significance was further interpreted based on the ‘Prob > F’ values, where values lower than 0.05 indicate statistical significance, whereas values greater than 0.1 represent insignificant model parameters. The initial ANOVA results suggested that both response models (Y1 and Y2) were statistically insignificant.

A developed model is typically considered reliable when the lack-of-fit result is statistically insignificant ($P > 0.05$), suggesting good agreement between the predicted and experimental data (Esmi et al., 2023). In ANOVA analysis, the lack-of-fit test is used to determine the suitability of the regression model within the investigated experimental range. Significant lack of fit may indicate that the model is unable to adequately represent the experimental behaviour due to unaccounted variations or insufficient regression fitting (Favier et al., 2023; Kadier et al., 2022). As shown in Table 4, the initial models for Y1 and Y2 exhibited significant lack-of-fit behaviour, with F-values of 110.34 and 75.00 and associated probability values of 0.0003 and 0.0004, respectively. This initial analysis indicated that the full quadratic model was insufficient ($P < 0.05$ for lack of fit). To improve accuracy, a stepwise algorithm was employed to select significant cubic terms and eliminate insignificant interactions. This resulted in a refined model that demonstrated high significance ($P < 0.0001$) and a non-significant lack of fit, making it suitable for optimisation (Favier et al., 2023). The final empirical quadratic equations describing the response functions are presented in Equations 10 and 11.

$$\begin{aligned} \text{Colour removal (\%)} (Y_1) = & 51.07 - 5.22X_1 - 4.18X_2 + 5.17X_1X_2 \\ & + 16.25X_1^2 + 17.37X_2^2 + 11.44 X_1^2X_2 \\ & - 5.07 X_1X_2^2 \end{aligned} \quad [10]$$

$$\begin{aligned} \text{Normalised flux (Y}_2\text{)} = & 0.5601 - 0.1518X_1 + 0.1309X_2 \\ & - 0.0342X_1X_2 - 0.0210X_1^2 + 0.0041X_2^2 \\ & - 0.1149 X_1^2X_2 - 0.2219 X_1X_2^2 \end{aligned} \quad [11]$$

The final reduced cubic models for Y_1 and Y_2 incorporated two linear terms (X_1 and X_2), a second-order interaction term (X_1X_2), two quadratic terms (X_1^2 and X_2^2), and two cubic interaction terms ($X_1^2X_2$ and $X_1X_2^2$). The statistically significant parameters identified after stepwise regression analysis are presented in Table 4, where all retained terms satisfied the significance requirement of $p < 0.05$. For colour removal (Y_1), all model terms were statistically significant, with X_2^2 representing the dominant contributor to the ANOVA model. Meanwhile, the significant contributors for normalised flux (Y_2) were limited to X_1 , X_2 , $X_1^2X_2$, and $X_1X_2^2$. Model reduction was performed to eliminate insignificant terms and improve the adequacy of the regression models. The results of the analysis of variance for the modified response variables are presented in Table 5. The F-values of the adjusted models were substantially higher and accompanied by lower P-values, indicating improved model significance and predictive capability. High F-values of 764.31 and 192.69 for Y_1 and Y_2 , respectively, together with probability values below 0.0001, confirmed the adequacy and statistical significance of the developed models (Ecer et al., 2024). The resulting lack-of-fit and probability values further supported the predictive reliability of the regression models.

Table 4
ANOVA results for the investigated response variables

Source	Colour Removal (%) Response						Contribution (%)
	Sum of Square	df	Mean Square	F-value	P-value (Prob>F)	Remarks	
Model	4091.19	5	818.24	18.05	0.0007	Not significant	
X_1	481.18	1	481.18	10.62	0.0139		10.59
X_2	19.06	1	19.06	0.4205	0.5374		0.42
X_1X_2	107.02	1	107.02	2.36	0.1683		2.35
X_1^2	1837.24	1	1837.24	40.54	0.0004		40.43
X_2^2	1837.24	1	1837.24	46.33	0.0003		46.21
Lack of fit	313.47	3	104.49	110.34	0.0003	Significant	
Source	Normalised Flux Response						Contribution (%)
	Sum of Square	df	Mean Square	F-value	P-value (Prob>F)	Remarks	
Model	0.6036	5	0.1207	6.62	0.0139	Not significant	
X_1	0.5524	1	0.5524	30.30	0.0009		91.54
X_2	0.0431	1	0.0431	2.37	0.1680		7.16
X_1X_2	0.0047	1	0.0047	0.2566	0.6280		0.78
X_1^2	0.0031	1	0.0031	0.1683	0.6939		0.51
X_2^2	0.0001	1	0.0001	0.0066	0.9377		0.02
Lack of fit	0.1254	3	0.0418	75.00	0.0004	Significant	

Table 5
ANOVA results for the modified response variable

Source	Colour Removal (%) Response						Contribution (%)
	Sum of Square	df	Mean Square	F-value	P-value (Prob>F)	Remarks	
Model	4404.34	7	629.19	764.31	<0.0001	Significant	
X ₁	108.93	1	108.93	132.32	<0.0001		2.40
X ₂	69.74	1	69.74	84.71	0.0003		1.54
X ₁ X ₂	107.02	1	107.02	130.00	<0.0001		2.36
X ₁ ²	1837.24	1	1837.24	2231.80	<0.0001		40.50
X ₂ ²	2099.81	1	2099.81	2550.75	<0.0001		46.29
X ₁ ² X ₂	261.65	1	261.55	317.85	<0.0001		5.77
X ₁ X ₂ ²	51.49	1	51.49	62.55	0.0005		1.14
Lack of fit	0.3280	1	0.3280	0.3464	0.5878	Not significant	

Source	Normalised Flux Response						Contribution (%)
	Sum of Square	df	Mean Square	F-value	P-value (Prob>F)	Remarks	
Model	0.7286	7	0.1041	192.69	<0.0001	Significant	
X ₁	0.0922	1	0.0922	170.68	<0.0001		31.41
X ₂	0.0685	1	0.0685	126.86	<0.0001		23.25
X ₁ X ₂	0.0047	1	0.0047	8.66	0.0321		1.59
X ₁ ²	0.0031	1	0.0031	5.68	0.0629		1.05
X ₂ ²	0.0001	1	0.0001	0.2215	0.6577		0.04
X ₁ ² X ₂	0.0264	1	0.0264	48.91	0.0009		9.00
X ₁ X ₂ ²	0.0985	1	0.0985	182.38	<0.0001		33.56
Lack of fit	0.0005	1	0.0005	0.8455	0.4099	Not significant	

Model Adequacy

Table 6 presents the coefficient of determination (R²) values used to assess the adequacy of the developed regression models. The R² parameter reflects the degree of agreement between the predicted and experimental data by representing the ratio of explained variation to total variation (Zhou et al., 2023). A model is generally considered to exhibit better fitting performance when the R² value approaches 1 (Favier et al., 2023). In this study, high R² values of 0.9991 for colour removal and 0.9963 for normalised flux confirmed excellent agreement between the predicted and observed responses. The good agreement between the experimental and predicted data was obtained for the developed models, as shown in Figures 5(a) and 6(a). This behaviour could be attributed to the entrapment of MO dye molecules in the void spaces of the fibril network, which resulted in a lower permeate transport rate but higher dye retention. Also, the difference in adjusted R² and predicted R² for both responses was less than 0.2, which was acceptable for the developed models

to describe the filtration performance. The colour removal model produced adjusted and predicted R^2 values of 0.9978 and 0.9939, respectively, whereas the normalised flux model recorded values of 0.9911 and 0.9540.

Residual analysis was further used to evaluate the deviation between the experimental and predicted responses. The close agreement between predicted R^2 and adjusted R^2 values confirmed the adequacy and reliability of the developed model within the investigated operating range. The normal probability plots shown in Figures 5b and 6b demonstrated that the residuals were randomly distributed and followed an approximately linear trend, indicating normally distributed and statistically insignificant model errors. Externally studentised residuals were calculated by normalising the residual values using the estimated standard deviation to identify potential outlier observations. One outlier was detected at a pH of 3 and a concentration of 450 mg/L MO dye. This behaviour might be associated with the electrostatic repulsion of the CNF filter paper under low ZPC conditions. In this case, both the membrane surface and MO dye molecules were positively charged, and this decreased the interaction between them. The distribution of the residuals in Figure 5c and Figure 6c was random, which indicates that the regression model was appropriate to predict the behaviour of colour removal and normalised flux. Furthermore, the residual plots against the experimental runs in Figures 5d and 6d revealed that the standardised residual values were within the acceptable range of ± 2 , which showed that the quadratic response surface model is reasonably fitted.

Significant precision analysis was also carried out to assess the reliability of the developed regression model by considering the signal-to-noise ratio. Sidik et al. (2019) suggest that the values above 4 are considered acceptable model performances. The adequate precision value of colour removal was 64.6398, and the normalised flux was 44.7517 in this study. These values are indicative of a good response of the signal and foretell that the built model was appropriate for navigating the design space.

Table 6
Summary of RSM model fitting for the response variables

Response (Y ₁): Colour Removal (%)							
Mean	Std. dev.	R ²	Adj. R ²	Pred. R ²	C.V.	A.P.	PRESS
71.76	0.9073	0.9991	0.9978	0.9939	1.26	64.64	26.91
Response (Y ₂): Normalised Flux							
Mean	Std. dev.	R ²	Adj. R ²	Pred. R ²	C.V.	A.P.	PRESS
0.5497	0.0232	0.9963	0.9911	0.9540	4.23	44.75	0.0336

Note. Std. dev. = standard deviation; R^2 = coefficient of determination; Adj. R^2 = adjusted R^2 ; Pred. R^2 = predicted R^2 ; C.V. = coefficient of variance; A.P. = adequate precision and PRESS = predicted residual error sum of square

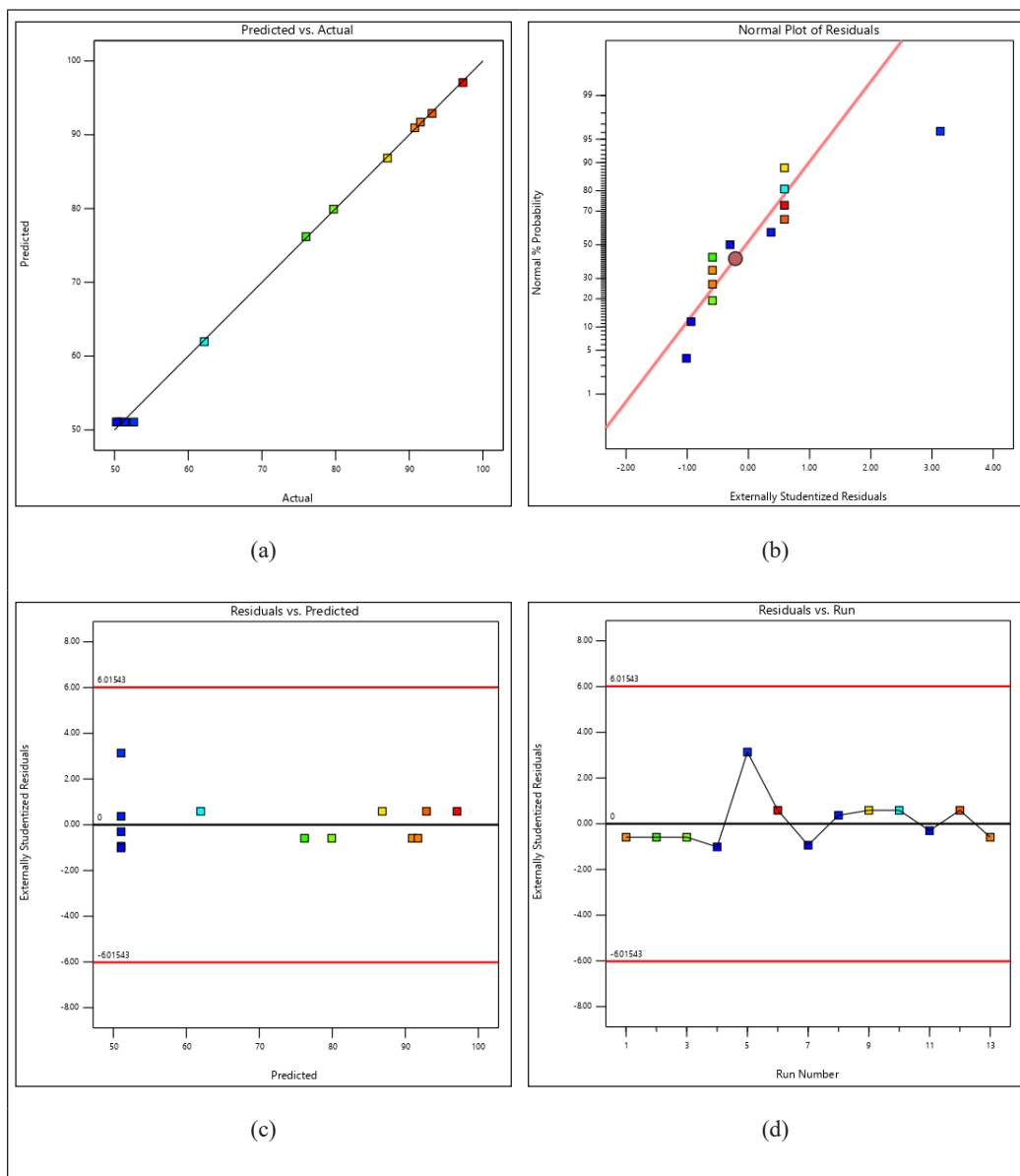


Figure 5. Residual diagnostic plots for the colour removal response consisting of (a) predicted versus actual response values; (b) normal probability plot of externally studentised residuals; (c) predicted residuals against externally studentised residuals; and (d) externally studentised residuals plotted against experimental runs

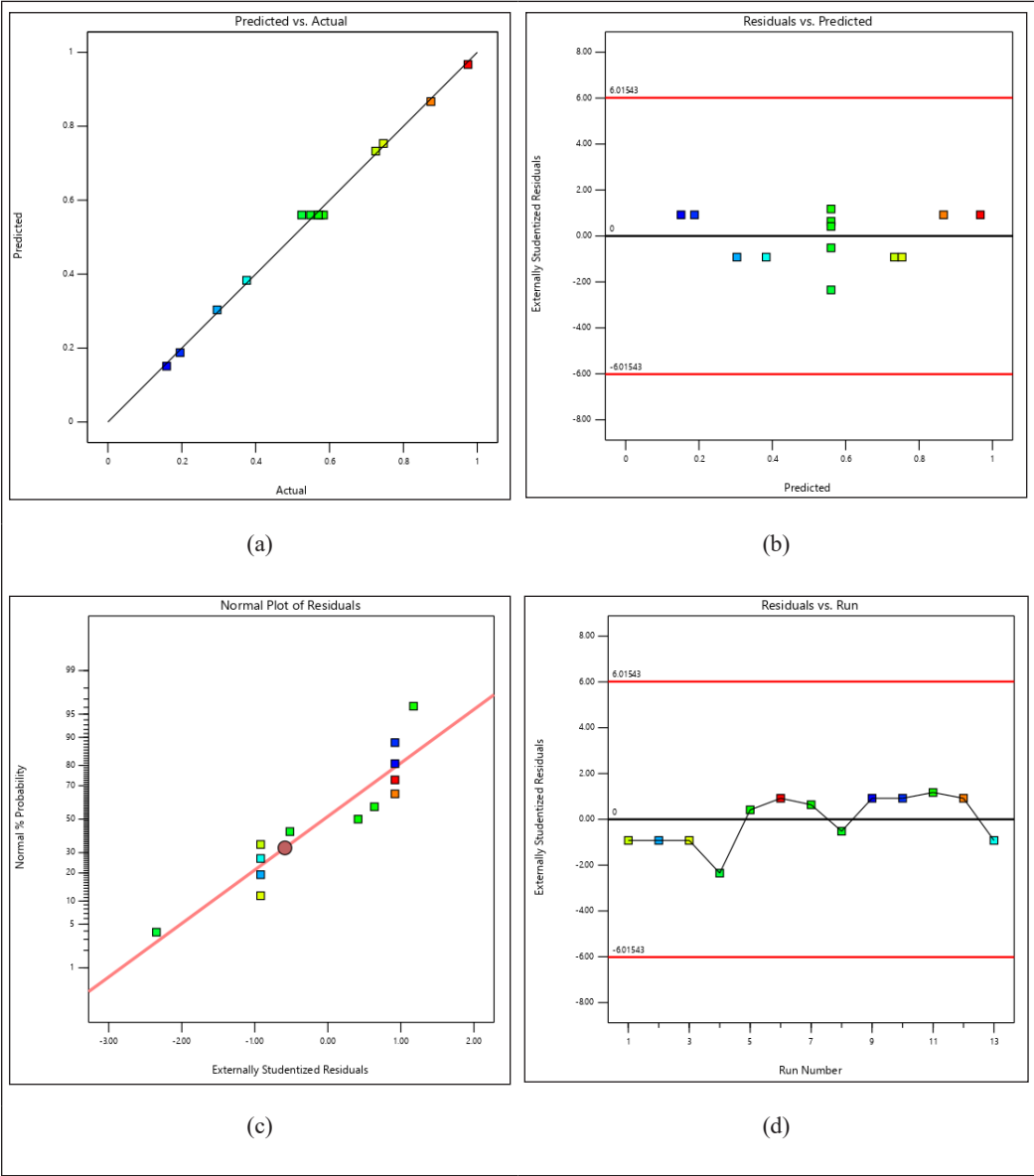


Figure 6. The normalised flux response is shown in the following plots using the residue analysis: (a) predicted vs experimental; (b) normal probability plot of externally studentised residuals; (c) predicted residuals vs externally studentised residuals; and (d) externally studentised residuals vs experimental runs

Influence of Independent Variables on Colour Removal and Normalised Flux Using CCD-RSM

The solution pH changed the surface charge properties of the CNF filter paper and the interaction between the MO dye molecules and the filter paper during filtration. The maximum colour removal efficiency (Y1) was found in an acidic medium. The pH removal efficiency gradually declined with increasing pH towards 6.5 and rose slightly at alkaline pH. The normalised flux (Y2), on the other hand, presented a negative trend as the pH increased. Accumulation of the MO dye on the surface of the filter paper during the filtration process had a close relationship with the filtration behaviour. The permeate flux was then more stable, which demonstrated the formation of a secondary fouling layer on the surface of the CNF filter paper (Satyanarayana et al., 2005).

The interaction between the solution pH and the isoelectric point (IEP) of the prepared filter paper was associated with the filtration performance. The IEP of the CNF filter paper was about 8.10, thus the surface was still positive at the pH levels below 8.1. This situation helped the electrostatic attraction of MO dye molecules (IEP ~3.47), which led to better removal of the dye under acidic conditions (Das et al., 2021). The interaction between the charged dye molecules and the filter paper surface was also shown in changes of flux behaviour. Chemical adsorption and interactions between the functional groups of the CNF structure could have played a role in the filtration behaviour, at higher pH conditions (Bandini & Mazzoni, 2005). Under strongly alkaline conditions (pH 9-10), however, the permeate flux is further reduced by electrostatic repulsion between the negatively charged dye molecules and the surface of the filter paper. This repulsion caused dye aggregation, and some of these dye aggregates were found to penetrate the void spaces and fibre network of the CNF filter paper.

The concentration of dye was also found to influence the filtration of the CNF filter paper. The increase in dye concentration was an indirect cause of flux reduction because it created a higher osmotic pressure inside the system (Hairom et al., 2014). For the colour removal response (Y1), two different trends were observed as the initial concentration of the MO dye was increased. As the concentration of the dye was raised, the removal efficiency decreased from about 83.10% to 72.60% before it gradually increased to 97.28% at 600 mg/L. The normalised flux, on the other hand, increased slightly in the intermediate concentration region. This behaviour may be linked to the interaction of the combined effect of osmotic pressure, dye adsorption, and electric double layer interaction on the filter paper's surface (Desa et al., 2019). Meanwhile, large dye accumulations on the filter paper surface resulted in more pronounced fouling behaviour in filtration.

Surface and Contour Plot Analysis

The response surfaces plotted in three dimensions (3D) show the interaction between the operating variables and the responses measured as peaks and curvature of the plots. Contour plots were drawn to show the influence of the combination of two independent variables - solution pH and initial dye concentration at three operating levels.

Figures 7a-d demonstrate the effect of initial MO dye concentration on solution pH for the CNF filter paper composition of 50:50. Response surfaces are shown that represent the filtration performance post 3 hours of operation in the crossflow filtration system for colour removal and normalised flux responses.

It can be observed in Figure 7a that under acidic conditions, the colour removal efficiency was higher at high dye concentration. The removal performance was also good with a combination of low dye concentration and alkaline pH, but moderate operating conditions yielded low removal efficiency. In the acidic pH range (4-6.5), the removal efficiency decreased from ~97.38% to 72.57% as the dye concentration varied from high to intermediate. The response surface at higher pH values (6.5-9) and dye concentrations (450-600mg/l) showed a more pronounced curvature, with colour removal gradually improving from 72.58% to 90.04%. The same pattern of behaviour is seen with the darker red area in the contour plot of Figure 7(b) representing higher response values. The optimum condition for MO dye removal based on the RSM optimisation analysis was achieved by using an initial dye concentration of 600mg/L at pH 4, which resulted in a 97.28% efficiency of colour removal.

It was found that higher colour removal in both acid and alkaline conditions might be associated with the aggregation of dye molecules during filtration. Possibly, the aggregated dye particles benefited with regard to their retention in the CNF filter paper in the interfibre bonding regions and void spaces. With the passage of time, during filtration, the deposited MO dye layers started to form on the surface of the filter paper and a secondary layer was formed, which contributed to a higher colour removal efficiency.

The response surface and contour plots of the normalised flux response are shown in Figures 7c and 7d, respectively. The normalised flux value increased (0.9748-0.9751) at higher dye concentration (540 to 600 mg/L) under acidic condition as shown by the darker region in the contour plot. Lower responses to the flux, however, were primarily seen around pH 6.5 and dye concentrations of 300 to 450 mg/L with normalised flux ranging from 0.2738 to 0.2869. Additional flux reductions were observed at pH 9 under 600mg/l dye concentration and at pH 4 under 300mg/l dye concentration with normalised flux value of 0.0589 and 0.0745 respectively.

The observed behaviour can be associated with the formation of a compact dye deposition layer on the CNF filter paper surface. This accumulated layer functioned as a secondary filtration barrier, limiting the transport of treated dye solution through

the filter and subsequently reducing the normalised flux. The flux response in this study displayed an unstable trend similar to the colour removal behaviour, particularly as the pH and initial dye concentration increased. Similar findings were reported by Ricceri et al. (2022), who investigated fouling behaviour in membrane distillation systems optimised using CCD with inlet temperature and crossflow velocity as operating variables. Their study showed that fouling formation did not exhibit a consistent relationship with the investigated variables, resulting in unstable flux decline behaviour caused by variations in fouling layer thickness on the membrane surface.

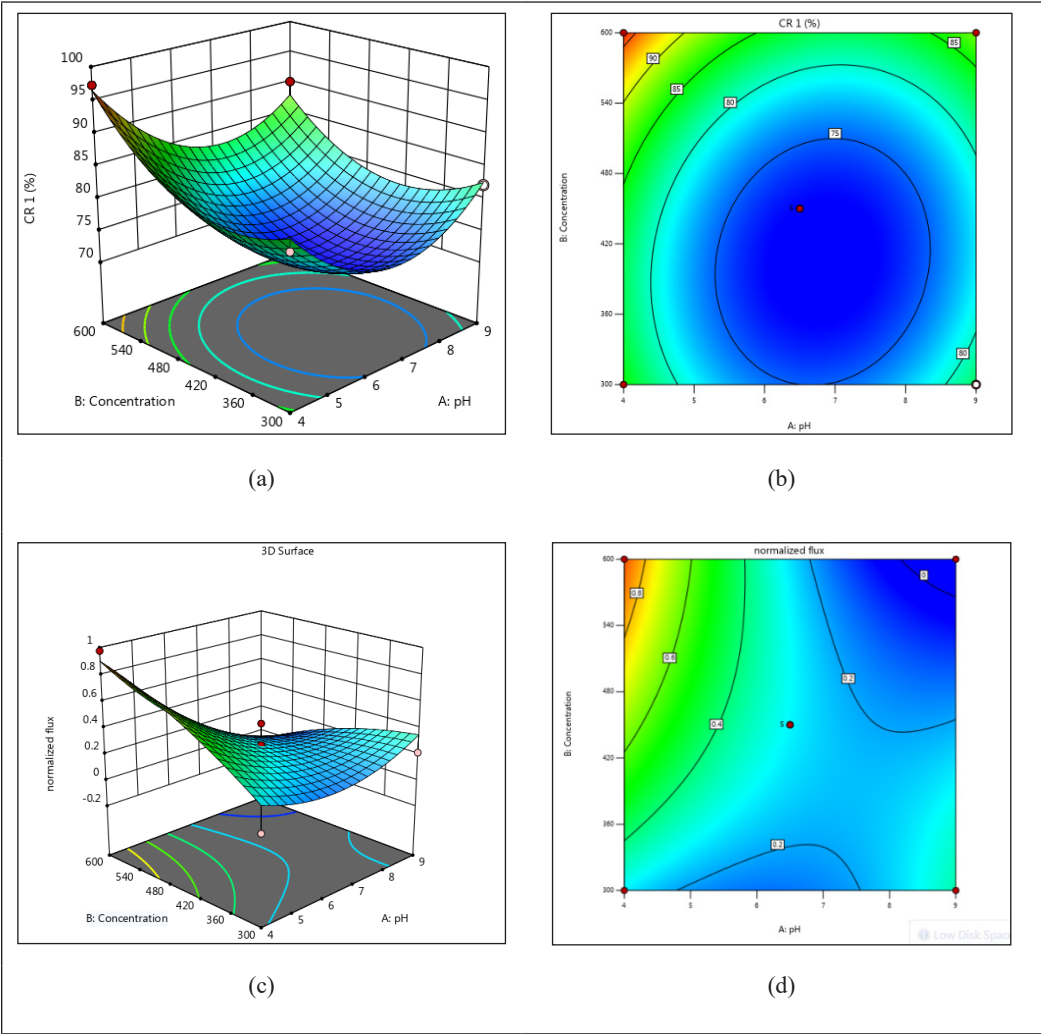


Figure 7. 3D response surface and contour plots showing the combined effects of initial MO dye concentration and solution pH on (a, b) colour removal efficiency and (c, d) normalised flux for the 50:50 CNF filter paper composition

Model Optimisation and Validation

The objective of the optimisation procedure was to identify the optimum operating conditions from the experimental design for achieving the targeted filtration responses (Veza et al., 2023). A desirability function method was employed to simultaneously maximise colour removal percentage and normalised flux. The desirability function ranges from 0 to 1, where 0 indicates responses outside the acceptable range and 1 represents complete fulfilment of the optimisation objective (Ebba et al., 2022; Saeed et al., 2015). The CCD-RSM model predicted optimum conditions at pH 4 and 600 mg/L initial MO dye concentration using a 50:50 cellulose dosage ratio. At these conditions, the predicted responses were 97.28% colour removal and 0.9748 normalised flux. Experimental validation was subsequently performed in triplicate to confirm the reliability of the optimised model predictions.

The fabricated *Neolamarckia cadamba* CNF filter paper was compared with different nanocellulose based materials reported in literature. The developed filter paper demonstrated a similar effectiveness to filters based on wood cellulose, as reported by Mautner and Bismarck (2021) in regard to the ability to reject dyes. But the current system showed higher flux holding potential during filtration. It was previously reported that wood pulp derived membranes and bacterial cellulose networks were able to reach high rejection percentages around 95%~98%, however, they were generally operated under the dead-end filtration mode, and the permeabilities of the membranes were very low. Similarly, bamboo-derived cellulose materials and adsorbents with agricultural waste also showed good dye removal efficiencies from 88% to 92.4%. However, many of these systems were constrained by low density or batch adsorption process rather than a continuous filtration process (Wu et al., 2017).

The optimum operating conditions and corresponding predicted responses for colour removal and normalised flux for MO dye treatment are summarised in Table 7. Experimental data showed good agreement with the model-predicted values, which suggested the developed CCD-RSM model was adequate to describe the performance of the CNF filter paper for the crossflow filtration system. Moosazade et al. (2021) studied the removal of Pb from water using CCD-RSM optimisation in an electrocoagulation-flotation system and obtained a maximum removal efficiency of ~97.28% at an electrolysis time of 77.65 min and a current of 0.9 A. The optimisation method used in this study also demonstrated good ability to optimise the operating conditions of textile wastewater treatment with minimum difference between the predicted and experimental responses as reported by Hermosilla et al. (2009).

Table 7
Optimum conditions and laboratory experiment validation

Response (Y ₁): Colour Removal (%)					
Run	pH	Initial MO dye Concentration (mg/L)	Experimental Condition	Predicted Condition	Error (%)
1	4	600	97.28	96.79	-0.51
2	4	600	96.31	96.79	0.50
3	4	600	96.78	96.79	0.01
Response (Y ₂): Normalised Flux					
Run	pH	Initial MO dye Concentration (mg/L)	Experimental Condition	Predicted Condition	Error (%)
1	4	600	0.9748	0.9801	0.54
2	4	600	0.9874	0.9801	-0.74
3	4	600	0.9781	0.9801	0.20

To test the predictive ability of the developed model, six validation experiments were carried out with operating conditions not covered in the original CCD data. It has been reported that a few experimental points may be enough to validate and do not necessarily represent the predictive ability of the developed model (Hamid et al., 2016). So, six operating conditions with different unseen factors were chosen to obtain a more comprehensive evaluation of the model applicability, as shown in Table 8. These validation runs were done inside and outside the range of the original experiments. Good agreement was seen between the predicted and experimental results for the residual values obtained for both responses studied. In general, the RSM models could predict the responses in the original CCD experiments and the additional validation experiments with acceptable accuracy.

Table 8
Validation of the developed model using unseen experimental conditions

Response (Y ₁): Colour Removal (%)						
Run	pH	Initial MO dye Concentration (mg/L)	Cellulose Dosage	Experimental	Predicted	Residual Error %
1	3	100	60:40	85.34	87.04	-1.99
2	7	100	70:30	86.78	88.76	-2.28
3	10	100	80:20	90.10	89.98	0.13
4	3	300	60:40	62.11	63.05	-1.51
5	7	300	70:30	71.00	69.78	1.72
6	10	300	80:20	75.49	77.32	-2.42

Table 8 (continued)

Response (Y ₂): Normalised Flux						
Run	pH	Initial MO dye Concentration (mg/L)	Cellulose Dosage	Experimental	Predicted	Residual Error %
1	3	100	60:40	0.1400	0.1387	0.93
2	7	100	70:30	0.9664	0.9867	-2.10
3	10	100	80:20	0.3211	0.3512	-9.37
4	3	300	60:40	0.2687	0.2500	6.96
5	7	300	70:30	0.9037	0.9157	-1.33
6	10	300	80:20	0.1251	0.131	-6.39

Membrane Fouling Mechanism Analysis

The membrane fouling behaviour under the optimum synthetic dye treatment conditions using the CNF filter paper in the crossflow filtration system was analysed in this study. The fitting profile of the obtained data by the Wiesner and Aptel equations is presented in Figure 8, and the respective R² values and fitted rate constants (K) are summarised in Table 9 for fouling model evaluation.

Analysis revealed that cake layer formation was the major fouling mechanism in the first and second stages of filtration, with R² values of 0.9528 and 0.9456, respectively. The observed gel/cake layer developed from the accumulation and coagulation of pollutant molecules retained on the CNF filter paper surface. Progressive deposition of these foulants reduced the permeate flux throughout the filtration process while improving the overall colour removal efficiency. This behaviour is consistent with the optimum filtration performance obtained in this study, which achieved 97.28% colour removal and a normalised flux of 0.9748. The sharp decrease in permeate flux observed at the early stage of filtration further suggested rapid development of the cake layer as the primary fouling mechanism. Similar observations have been reported in previous nanofiltration studies, where cake formation was identified as the primary contributor to flux decline behaviour (Sidik et al., 2019). The fouling behaviour may be associated with the larger size of MO dye particles relative to the void spaces within the CNF fibre network, promoting dye deposition and dense cake layer formation on the filter paper surface (Cerón-Vivas et al., 2019; Zhang et al., 2021). However, the complete blocking, intermediate blocking, and standard blocking models showed weak agreement with the experimental data for both filtration stages, with R² values lower than 0.9. These findings suggest that the membrane fouling behaviour under optimum conditions followed a two-step cake layer formation mechanism.

Table 9
Rate constant and correlation of determination (R^2) values for CNF filter paper fouling mechanisms at optimum conditions

Blocking Filtration Laws	CNF Filter Paper (50:50)			
	Stage 1		Stage 2	
	R^2	$K \text{ (s}^{-1}\text{)}$	R^2	$K \text{ (s}^{-1}\text{)}$
Complete Blocking	< 0.1	< 0.1	< 0.1	< 0.1
Intermediate Blocking	0.7391	7.257	0.3539	3.544
Standard Blocking	0.3864	2.475	< 0.1	0.9054
Cake Formation	0.9528	34.80	0.9456	34.03

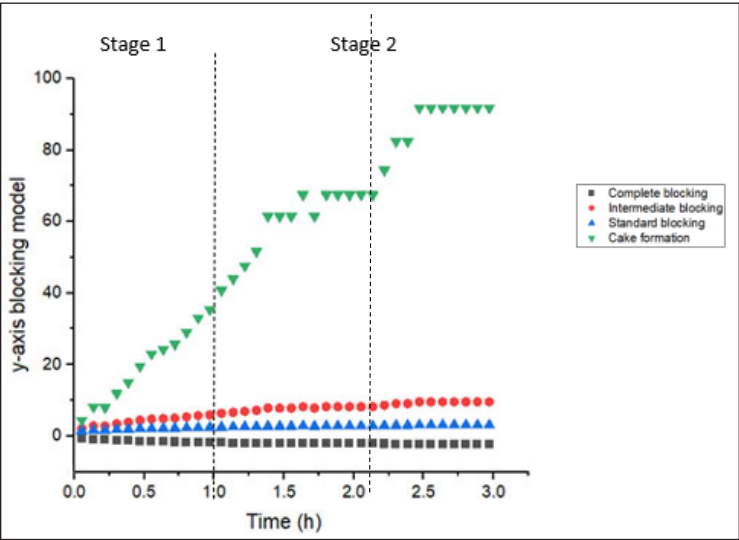


Figure 8. Fouling mechanism analysis of CNF filter paper under optimum filtration conditions (50:50 cellulose dosage ratio, pH 4, and 600 mg/L initial MO dye concentration)

CHALLENGES AND FUTURE PERSPECTIVES OF CNF FILTER PAPER

The practical application of *Neolamarckia cadamba* CNF filter paper in industrial textile wastewater treatment is supported by its economic and operational feasibility. From an economic perspective, *Neolamarckia cadamba* (locally known as Kelempayan) is a fast-growing Malaysian plantation species that provides a sustainable and low-cost feedstock compared to synthetic polymers. However, there were limitations in terms of the durability and resilience of the fabricated nanopapers during extended contact times. There is a need for further advances in the production of CNF filter papers with enhanced mechanical stability and durability of the nanocellulose fibre network. It is still essential to maintain an appropriate density and porosity for adequate filtration.

The fabricated filter paper may also have better reusability potential due to better structural stability.

CONCLUSION

This work aimed to study the optimisation of MO dye removal by the CNF filter paper in the crossflow filtration system. CCD and RSM were used to identify the conditions that resulted in high colour removal and acceptable normalised flux performance. The results indicated that the pH of the solution, and the initial dye concentration of MO significantly influenced the filtration behaviour of the prepared CNF filter paper. The optimum operating condition was determined as a 50:50 cellulose dosage ratio, an initial MO dye concentration of 600 mg/L and a pH of 4 from the optimisation analysis under high desirability conditions. These developed quadratic models exhibited good correlations with the experimental data, with R^2 of 0.9991 for colour removal and R^2 of 0.9963 for normalised flux. The maximum colour removal (97.28%) and normalised flux (0.9748) of the crossflow filtration system were obtained under optimum conditions. Fouling analysis showed that fouling during filtration was primarily attributed to the formation of a cake layer, which primarily occurred due to the interaction of the MO dye solution with the surface of the CNF filter paper. The results of the overall fabricated *Neolamarckia cadamba* CNF filter paper indicated that it is a potential sustainable filtration material for industrial wastewater treatment applications with good filtration behaviour and high removal performance.

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REFERENCES

- Amin, I. N. H. M., & Nizam, M. H. M. (2016). Assessment of membrane fouling indices during removal of reactive dye from batik wastewater. *Journal of Water Reuse and Desalination*, 6(4), 505-514. <https://doi.org/10.2166/wrd.2016.072>
- Aryanti, N., Saraswati, A., Pratama Putra, R., Nafiunisa, A., & Hesti Wardhani, D. (2018). Fouling mechanism of micelle enhanced ultrafiltration with SDS surfactant for indigozol dye removal. *Jurnal Teknologi*, 80(3), 101-108. <https://doi.org/10.11113/jt.v80.12741>
- Bai, Y., Wu, Y. H., Wang, R. N., Xue, S., Chen, Z., & Hu, H. Y. (2023). Critical minority fractions causing membrane fouling in reclaimed water: Fouling characteristics, mechanisms and control strategies. *Environment International*, 173, Article 107818. <https://doi.org/10.1016/j.envint.2023.107818>

- Cerón-Vivas, A., Cáceres, K. T., Rincón, A., & Cajigas, A. (2019). Influence of pH and the C/N ratio on the biogas production of wastewater. *Revista Facultad de Ingeniería*, 92, 88-95. <https://doi.org/10.17533/udea.redin.20190627>
- Das, L., Das, P., Bhowal, A., & Bhattacharjee, C. (2021). Enhanced biosorption of fluoride by extracted nanocellulose/polyvinyl alcohol composite in batch and fixed-bed system: ANN analysis and numerical modelling. *Environmental Science and Pollution Research*, 28(34), 47107-47125. <https://doi.org/10.1007/s11356-021-14026-x>
- Desa, A. L., Hairom, N. H. H., Ng, L. Y., Ng, C. Y., Ahmad, M. K., & Mohammad, A. W. (2019). Industrial textile wastewater treatment via membrane photocatalytic reactor (MPR) in the presence of ZnO-PEG nanoparticles and tight ultrafiltration. *Journal of Water Process Engineering*, 31, Article 100872. <https://doi.org/10.1016/j.jwpe.2019.100872>
- Ebba, M., Asaithambi, P., & Alemayehu, E. (2022). Development of electrocoagulation process for wastewater treatment: optimisation by response surface methodology. *Heliyon*, 8(5), Article e09383. <https://doi.org/10.1016/j.heliyon.2022.e09383>
- El-Sheekh, M. M., El-Nagar, A. A., ElKelawy, M., & Bastawissi, H. A. E. (2023). Maximisation of bioethanol productivity from wheat straw, performance and emission analysis of diesel engine running with a triple fuel blend through response surface methodology. *Renewable Energy*, 211, 706-722. <https://doi.org/10.1016/j.renene.2023.04.145>
- Esmi, F., Dalai, A. K., & Hu, Y. (2023). Optimisation and kinetic studies of 12-tungstophosphoric supported mesoporous aluminosilicate through response surface methodology for biodiesel production using green seed canola oil. *Fuel*, 348, Article 128594. <https://doi.org/10.1016/j.fuel.2023.128594>
- Favier, L., Andrei-Ionuț Simion, Hlihor, R. M., Fekete-Kertész, I., Molnár, M., Harja, M., & Vial, C. (2023). Intensification of the photodegradation efficiency of an emergent water pollutant through process conditions optimisation by means of response surface methodology. *Journal of Environmental Management*, 328, Article 116928. <https://doi.org/10.1016/j.jenvman.2022.116928>
- Hairom, N. H. H., Mohammad, A. W., & Kadhum, A. A. H. (2014). Nanofiltration of hazardous Congo red dye: Performance and flux decline analysis. *Journal of Water Process Engineering*, 4, 99-106. <https://doi.org/10.1016/j.jwpe.2014.09.008>
- Hairom, N. H. H., Mohammad, A. W., & Kadhum, A. A. H. (2015). Influence of zinc oxide nanoparticles in the nanofiltration of hazardous Congo red dyes. *Chemical Engineering Journal*, 260, 907-915. <https://doi.org/10.1016/j.cej.2014.08.068>
- Hamid, H. A., Jenidi, Y., Thielemans, W., Somerfield, C., & Gomes, R. L. (2016). Predicting the capability of carboxylated cellulose nanowhiskers for the remediation of copper from water using response surface methodology (RSM) and artificial neural network (ANN) models. *Industrial Crops and Products*, 93, 108-120. <https://doi.org/10.1016/j.indcrop.2016.05.035>
- Hanafy, H. (2021). Adsorption of methylene blue and bright blue dyes on bayleaf capertree pods powder: Understanding the adsorption mechanism by a theoretical study. *Journal of Molecular Liquids*, 332, Article 115680. <https://doi.org/10.1016/j.molliq.2021.115680>

- Hermia, J. (1985). Blocking Filtration. Application to non-Newtonian fluids. In A. Rushton (Ed.), *Mathematical models and design methods in solid-liquid separation* (pp. 83-89). Springer Netherlands. https://doi.org/10.1007/978-94-009-5091-7_5
- Hermosilla, D., Cortijo, M., & Huang, C. P. (2009). Optimising the treatment of landfill leachate by conventional Fenton and photo-Fenton processes. *Science of the Total Environment*, 407(11), 3473-3481. <https://doi.org/10.1016/j.scitotenv.2009.02.009>
- Hussain, S., Kamran, M., Khan, S. A., Shaheen, K., Shah, Z., Suo, H., Khan, Q., Shah, A. B., Rehman, W. U., Al-Ghamdi, Y. O., & Ghani, U. (2021). Adsorption, kinetics and thermodynamics studies of methyl orange dye sequestration through chitosan composites films. *International Journal of Biological Macromolecules*, 168, 383-394. <https://doi.org/10.1016/j.ijbiomac.2020.12.054>
- Janesch, J., Jones, M., Bacher, M., Kontturi, E., Bismarck, A., & Mautner, A. (2020). Mushroom-derived chitosan-glucan nanopaper filters for the treatment of water. *Reactive and Functional Polymers*, 146, Article 104428. <https://doi.org/10.1016/j.reactfunctpolym.2019.104428>
- Javaid, R., Qazi, U. Y., Ikhlaiq, A., Zahid, M., & Alazmi, A. (2021). Subcritical and supercritical water oxidation for dye decomposition. *Journal of Environmental Management*, 290, Article 112605. <https://doi.org/10.1016/j.jenvman.2021.112605>
- Jiang, Z., & Hu, D. (2019). Molecular mechanism of anionic dyes adsorption on cationized rice husk cellulose from agricultural wastes. *Journal of Molecular Liquids*, 276, 105-114. <https://doi.org/10.1016/j.molliq.2018.11.153>
- K, A., Mungray, A., Agarwal, S., Ali, J., & Chandra Garg, M. (2021). Performance optimisation of forward-osmosis membrane system using machine learning for the treatment of textile industry wastewater. *Journal of Cleaner Production*, 289, Article 125690. <https://doi.org/10.1016/j.jclepro.2020.125690>
- Kadier, A., Wang, J., Chandrasekhar, K., Abdeslahian, P., Islam, M. A., Ghanbari, F., Bajpai, M., Katoch, S. S., Bhagawati, P. B., Li, H., Kalil, M. S., Hamid, A. A., Abu Hasan, H., & Ma, P. C. (2022). Performance optimisation of microbial electrolysis cell (MEC) for palm oil mill effluent (POME) wastewater treatment and sustainable Bio-H₂ production using response surface methodology (RSM). *International Journal of Hydrogen Energy*, 47(34), 15464-15479. <https://doi.org/10.1016/j.ijhydene.2021.09.259>
- Latifah, J., Nurrul-Atika, M., Sharmiza, A., & Rushdan, I. (2020). Extraction of nanofibrillated cellulose from kelempayan (*Neolamarckia cadamba*) and its use as strength additive in papermaking. *Journal of Tropical Forest Science*, 32(2), <https://doi.org/10.26525/jtfs32.2.170>
- Mautner, A., & Bismarck, A. (2021). Bacterial nanocellulose papers with high porosity for optimised permeance and rejection of nm-sized pollutants. *Carbohydrate Polymers*, 251, Article 117130. <https://doi.org/10.1016/j.carbpol.2020.117130>
- Mautner, A., Kwaw, Y., Weiland, K., Mvubu, M., Botha, A., John, M. J., Mtibe, A., Siqueira, G., & Bismarck, A. (2019). Natural fibre-nanocellulose composite filters for the removal of heavy metal ions from water. *Industrial Crops and Products*, 133(March), 325-332. <https://doi.org/10.1016/j.indcrop.2019.03.032>
- Miao, R., Ma, B., Li, P., Wang, P., Wang, L., & Li, X. yan. (2021). Mitigation mechanism of ozonation in the casein fouling of ultrafiltration membranes: Possible application in dairy wastewater treatment. *Journal of Membrane Science*, 629, Article 119307. <https://doi.org/10.1016/j.memsci.2021.119307>

- Moosazade, M., Ashoori, R., Moghimi, H., Amani, M. A., Frontistis, Z., & Taheri, R. A. (2021). Electrochemical recovery to overcome direct osmosis concentrate-bearing lead: Optimisation of treatment process via RSM-CCD. *Water*, 13(21), Article 3136. <https://doi.org/10.3390/w13213136>
- Norrahma, S. S. A., Hamid, N. H. A., Hairom, N. H. H., Jasmani, L., & Sidik, D. A. B. (2023). Industrial textile wastewater treatment using *Neolamarckia cadamba* NFC filter paper via cross-flow filtration system. *Journal of Water Process Engineering*, 55, Article 104188. <https://doi.org/10.1016/j.jwpe.2023.104188>
- Petcu, A. R., Lazar, C. A., Rogozea, E. A., Olteanu, N. L., Meghea, A., & Mihaly, M. (2016). Nonionic microemulsion systems applied for removal of ionic dyes mixtures from textile industry wastewaters. *Separation and Purification Technology*, 158, 155-159. <https://doi.org/10.1016/j.seppur.2015.12.002>
- Pu, Y., Tang, J., Zeng, T., Hu, Y., Wang, Q., Huang, J., Pan, S., Wang, X. C., Li, Y., Hao Ngo, H., & Abomohra, A. (2022). Enhanced energy production and biological treatment of swine wastewater using anaerobic membrane bioreactor: Fouling mechanism and microbial community. *Bioresource Technology*, 362, Article 127850. <https://doi.org/10.1016/j.biortech.2022.127850>
- Ricceri, F., Blankert, B., Ghaffour, N., Vrouwenvelder, J. S., Tiraferri, A., & Fortunato, L. (2022). Unravelling the role of feed temperature and cross-flow velocity on organic fouling in membrane distillation using response surface methodology. *Desalination*, 540, Article 115971. <https://doi.org/10.1016/j.desal.2022.115971>
- Saeed, M. O., Azizli, K., Isa, M. H., & Bashir, M. J. K. (2015). Application of CCD in RSM to obtain optimised treatment of POME using Fenton oxidation process. *Journal of Water Process Engineering*, 8, e7-e16. <https://doi.org/10.1016/j.jwpe.2014.11.001>
- Satyanarayana, T., Raghukumar, C., & Shivaji, S. (2005). Extremophilic microbes: Diversity and perspectives. *Current Science*, 89(1), 78-90.
- Septevani, A. A., Rifathin, A., Sari, A. A., Sampora, Y., Ariani, G. N., Sudiarmanto, & Sondari, D. (2020). Oil palm empty fruit bunch-based nanocellulose as a super-adsorbent for water remediation. *Carbohydrate Polymers*, 229, Article 115433. <https://doi.org/10.1016/j.carbpol.2019.115433>
- Sidik, D. A. B., Hairom, N. H. H., & Mohammad, A. W. (2019). Performance and fouling assessment of different membrane types in a hybrid photocatalytic membrane reactor (PMR) for palm oil mill secondary effluent (POMSE) treatment. *Process Safety and Environmental Protection*, 130, 265-274. <https://doi.org/10.1016/j.psep.2019.08.018>
- Singh, M., Vajpayee, M., & Ledwani, L. (2021). Eco-friendly surface modification of natural fibres to improve dye uptake using natural dyes and application of natural dyes in fabric finishing: A review. *Materials Today: Proceedings*, 43, 2868-2871. <https://doi.org/10.1016/j.matpr.2021.01.078>
- Thiruppathi, K., Rangasamy, K., Ramasamy, M., & Muthu, D. (2021). Evaluation of textile dye degrading potential of ligninolytic bacterial consortia. *Environmental Challenges*, 4, Article 100078. <https://doi.org/10.1016/j.envc.2021.100078>
- Türgay, O., Ersöz, G., Atalay, S., Forss, J., & Welander, U. (2011). The treatment of azo dyes found in textile industry wastewater by anaerobic biological method and chemical oxidation. *Separation and Purification Technology*, 79(1), 26-33. <https://doi.org/10.1016/j.seppur.2011.03.007>

- Veza, I., Spraggon, M., Fattah, I. M. R., & Idris, M. (2023). Response surface methodology (RSM) for optimising engine performance and emissions fueled with biofuel: Review of RSM for sustainability energy transition. *Results in Engineering*, 18, Article 101213. <https://doi.org/10.1016/j.rineng.2023.101213>
- Wiesner, & Aptel. (1996). Mass transport and permeate flux and fouling in pressure-driven processes. In M. R. Wiesner & P. Aptel (Eds.), *Water treatment membrane conditions handbook*. McGraw-Hill.
- Wu, B., Geng, B., Chen, Y., Liu, H., Li, G., & Wu, Q. (2017). *Preparation and characteristics of TEMPO-oxidised cellulose nanofibrils from bamboo pulp and their oxygen-barrier application in PLA films*. *Frontiers of Chemical Science and Engineering*. 11. 1-10. <https://doi.org/10.1007/s11705-017-1673-8>
- Yadav, S., Yadav, A., Bagotia, N., Sharma, A. K., & Kumar, S. (2021). Adsorptive potential of modified plant-based adsorbents for sequestration of dyes and heavy metals from wastewater - A review. *Journal of Water Process Engineering*, 42, Article 102148. <https://doi.org/10.1016/j.jwpe.2021.102148>
- Yousefi, N., Jones, M., Bismarck, A., & Mautner, A. (2021). Fungal chitin-glucan nanopapers with heavy metal adsorption properties for ultrafiltration of organic solvents and water. *Carbohydrate Polymers*, 253, Article 117273. <https://doi.org/10.1016/j.carbpol.2020.117273>
- Zhang, W., Zhang, Y., Cao, J., & Jiang, W. (2021). Improving the performance of edible food packaging films by using nanocellulose as an additive. *International Journal of Biological Macromolecules*, 166, 288-296. <https://doi.org/10.1016/j.ijbiomac.2020.10.185>
- Zhou, H., Zhang, M., Chen, Q., Shan, Q., Liu, S., Lin, J., Ma, L., Zheng, G., Li, L., Zhao, C., Wei, L., Dai, X., & Yin, Y. (2023). Determination of amphenicol antibiotic residues in aquaculture products by response surface methodology modified QuEChERS method combined with UPLC-MS/MS. *Microchemical Journal*, 190, Article 108729. <https://doi.org/10.1016/j.microc.2023.108729>

Optimal Concentration and Application Timing of Teak Leaf Extract (*Tectona grandis*) as a Bioherbicide Against *Tridax procumbens* in Soybean

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ABSTRACT

Weed competition during soybean cultivation induces stress, leading to reduced yields. One of the dominant weeds in soybean cultivation is *Tridax procumbens*, and weed control is generally performed using herbicides, but can cause environmental pollution. Therefore, natural herbicides that are environmentally friendly and sustainable are required. Teak leaves, which contain allelopathy and are abundantly available in Indonesia, have the potential to be used as a bioherbicide. This study aimed to determine the optimal concentration and application time of teak leaf extract as a bioherbicidal agent against *T. procumbens* during soybean cultivation. The study was performed at the laboratory and greenhouse of the Faculty of Agriculture, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta. The research was conducted in two stages. The first stage was the germination stage, which used a complete randomised design with 10%, 20%, and 40% teak leaf extract concentrations. Meanwhile, the second stage was the initial growth stage using a complete randomised block design with treatments consisting of application 7 days pre-planting, planting time, and application 7 days post-planting. The 10% concentration of teak leaves was able to inhibit the germination force and vigour index of *T. procumbens* and did not inhibit soybean germination.

Therefore, a 10% teak leaf extract concentration was applied in the second stage, and optimal results at the time of application at planting were obtained because it was able to inhibit up to 100% of *T. procumbens* growth. However, further field-scale studies are needed to evaluate its practical application.

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Keywords: Allelopathy, bioherbicide, germination, seedling growth, soybeans, teak, *Tridax procumbens*

INTRODUCTION

Soybeans are one of Indonesia's preferred crops. In 2021, Indonesia's domestic soybean production was 613,300 tons, a 3.01% decrease from that of 2020. Furthermore, in 2023, soybean production declined to 576,300 tons or a decrease of 3.09% (Hulu, 2023). These declines in soybean production in Indonesia can be caused by reduced production area or constraints on soybean cultivation, such as biotic and abiotic stressors. One of these biotic stressors that inhibits soybean cultivation is weed competition.

Crop-weed competition alters how crops interact with light, space, water, and nutrients (Kaur et al., 2018). Weed competition can reduce soybean crop yield by up to 80% (Silva et al., 2015). Every gram of weed dry matter reduces crop yields because the weeds absorb one-third of the fertiliser nutrients during their early growth, including nitrogen, phosphorus, potassium, calcium, and magnesium (Kaur et al., 2018). One of the main weed species found in soybean production is *Tridax procumbens* (Rodrigues & Vivian, 2011). The impact of weeds on soybean crops necessitates weed control efforts to maintain high yields. Weed control can be performed mechanically or chemically. However, when used excessively, chemical control with herbicides can harm the biosphere by decreasing soil fertility, increasing pest populations, polluting the environment, and leading to resistant weed populations (Rajak et al., 2023).

Teak leaves contain allelopathic compounds. When teak leaves fall to the ground due to defoliation, these compounds can affect the growth of surrounding plants. Previous studies have shown that teak leaf extracts suppress the germination and growth of several plant species through allelopathic activity (Kato-Noguchi, 2021). The teak plant is widely planted in Yogyakarta, especially in the Gunung Kidul Regency. Teak plant leaves can be used as a bioherbicide because of their allelopathic properties and their abundant presence in the surrounding area. Using methanol-extracted teak leaf extract can reduce weed populations by 56% and inhibit the growth of *Echinochloa colona* and *Echinochloa crus-galli* (Kole et al., 2016).

Innovations in alternative weed control that are practical and environmentally friendly, such as bioherbicides, have been identified and attracted much interest. Moreover, the application of the leaf extract of cardoon (*Cynara cardunculus* L. var. *altilis* DC.) can inhibit the germination of four weeds, namely *Amaranthus retroflexus* L., *Portulaca oleracea* L., and *Stellaria media* (L.) Vill., and *Anagallis arvensis* L. (Scavo et al., 2020). Previous studies have also demonstrated the bioherbicidal potential of several plant extracts. Furthermore, *Cleome rutidosperma* contains toxic allelopathic compounds that can interfere with the germination of other weeds in rice cultivation, such as *Echinochloa crus-galli* (L.) P. Beauv., *Fimbristylis miliacea* (L.) Vahl, *Oryza sativa* f. *spontanea* Roshev, *Leptochloa chinensis* (L.) Nees, and *Cyperus iria* L. (Motmainna et al., 2024).

Total phenolic content was evaluated to estimate the potential allelopathic activity of teak leaf extract, as phenolic compounds are known to contribute to phytotoxic effects (Saludes-Zanfaño et al., 2024). Allelopathic effects can be evaluated through seed germination parameters (Li et al., 2024). Therefore, germination rate, germination index, and the number of abnormal sprouts were measured in this study. The root-stem ratio in soybean was evaluated to determine the relative sensitivity of root and stem growth to allelopathy (Xiao et al., 2024). Dry weight was measured as an indicator of the overall growth response of *T. procumbens* to teak leaf extract treatment (Respatie et al., 2024).

However, the effective concentration and optimal application timing of teak leaf extract remain unclear. Due to its allelopathic properties, teak leaf extract has potential as an environmentally friendly bioherbicide for weed management. Therefore, this study aimed to determine the effective concentration and application timing of teak leaf extract against weeds.

MATERIALS AND METHODS

Experimental Site

Fallen teak leaves were gathered from the experimental field of the Agricultural Faculty of Universitas Gadjah Mada, Banguntapan, Bantul District, Special Region of Yogyakarta Province, Indonesia (07°48'17" S, 110°24'45" E). Naturally fallen teak leaves were used in this research to support the utilisation of unused plant waste. The leaves were stored in dry conditions at room temperature before further processing. The germination experiment was conducted in November 2023 at the Plant Management Laboratory, and the field experiment was conducted from January to February 2024 at the greenhouse of the Agricultural Faculty of Universitas Gadjah Mada.

Experimental Design

The experimental design in the concentration and germination experiments was conducted using a single-factor, completely randomised design with four different concentrations (0, 10, 20, and 40%) and three replications. The extract concentrations were selected based on the study by Budianto et al. (2023), who reported that teak leaves contain approximately $4.3 \pm 0.15\%$ (w/w). The 10% concentration was assumed to represent the minimum inhibitory level because it contained the lowest proportion of phenolic compounds. Furthermore, the concentrations were increased to ensure a clear and significant difference between treatments and to evaluate the dose-dependent inhibitory effects of the extract.

Thereafter, a field experiment was conducted using a randomised complete block design with three treatments consisting of application 7 days pre-planting (D-7), planting time (D-0), and application 7 days post-planting (D+7), with four replications.

Extract and Solution Preparations

The teak leaves used were leaves that had fallen from the tree; therefore, they were already in a dry state. The teak leaves were ground into powder using a grinder. The powder was weighed according to the desired concentration: 10 g, 20 g, and 40 g of teak leaf powder were mixed with 100 mL of distilled water to prepare 10%, 20%, and 40% extracts, respectively. Therefore, the extraction ratio was maintained consistently at n g:100 mL for all treatments.

The mixtures were kept at room temperature (20-25 °C) for 24 h. After extraction, the mixtures were filtered through muslin cloth (<0.1 mm diameter) and then through Whatman No. 1 filter paper to obtain the final extracts. The extraction process was repeated for each experimental replicate to ensure reproducibility. All extracts were maintained at room temperature prior to application.

Determination of the Phenolic Content Equivalent to Gallic Acid

To determine the total phenol content equivalent to gallic acid, 50 mg of the sample was combined with 0.5 mL of Folin-Ciocalteu phenol reagent and 7.5 mL of distilled water. The mixture was shaken and allowed to stand for 10 min. Following this, 1.5 mL of 20% sodium carbonate solution was added. The mixture was shaken again and left for an additional 10 min. Finally, distilled water was added to make up a total volume of 10 mL, and the absorbance was measured at 760 nm using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Biochemical Analysis of the Teak Leaf Extract

Biochemical analysis of teak leaf extracts was performed by measuring the value of acidity (pH) and electrical conductivity (EC) of the teak leaf extracts with concentrations of 10%, 20%, and 40%, compared with the control (distilled water). The pH was measured by pH Tester HI189107 pHep, and EC are measured with an EC meter model number HI8731.

Bioassays

The experiment was arranged in a completely randomised design consisting of control treatment (A0) and teak leaf extract concentrations of 10% (A1), 20% (A2), and 40% (A3). Soybean and *T. procumbens* seeds were germinated separately in a petri dish lined with filter paper treated with 10 mL of teak leaf extract. Each treatment consisted of 10 seeds with three replications for each plant species. Seed germination was observed for 7 days.

Several germination parameters, including germination rate, germination index, and abnormal sprout formation, were evaluated to assess the effects of teak leaf extract on seed growth.

Sprouts were classified as abnormal if they had spiral hypocotyls and small primary roots. Below are the formulas used during this study, as shown in Equations 1 - 3:

$$\text{Germination Rate (\%)} = \left(\frac{\text{Number of Total Germinated Seeds}}{\text{Total of Number Seeds Tested}} \right) \times 100 \quad [1]$$

$$\text{Germination Index} = \sum \frac{G_t}{D_t} \quad [2]$$

where G_t denotes number of seeds germinated on day- t , and D_t corresponds to the Day of the count

$$\text{Abnormal Sprouts (\%)} = \left(\frac{\text{Number of Total Abnormal Sprouts}}{\text{Total of Number Germinated Seeds}} \right) \times 100 \quad [3]$$

Greenhouse experiment

The experiment was conducted under greenhouse conditions with temperatures ranging from 25-35 °C and relative humidity ranging from 50-90%. Soybean and *T. procumbens* were planted in 15 × 15 cm polybags containing 1L of sterilised soil media. Five soybean cultivars, *Anjasmoro* and 20 *T. procumbens* seeds, were planted in separate polybags. The 250 mL teak leaf extract solution was applied to each polybag at the specified treatment concentration. Three application treatment times were used in this study: before planting (D-7), at planting (D-0), and after planting (D+7). 250 mL of teak leaf extract was applied to the polybags. Watering was performed once per application time treatment. The variables measured at this stage were germination rate and dry weight. The following formula was used for the germination rate:

$$\text{Germination Rate (\%)} = \left(\frac{\text{Number of Total Germinated Seeds}}{\text{Total of Number Seeds Tested}} \right) \times 100 \quad [4]$$

Dry weights were measured by placing the plant parts into envelopes and oven-drying at 65 °C-85 °C for 48 h until a constant dry weight was obtained, then weighing.

The root/shoot ratio was calculated using the following formula:

$$\text{Root/Shoot Ratio} = \frac{\text{Root dry weight}}{\text{Shoot dry weight}} \quad [5]$$

Statistical Analysis

Data analysis was performed using analysis of variance with a significance level of 5% ($\alpha = 0.05$). If the data were significant, a post hoc test was used. Further tests for germination and growth were analysed using the Tukey test at the 5% significance level. All data analysis in this study was performed using R version 4.2.1.

RESULT AND DISCUSSION

Chemical Properties of the Teak Leaf Extract

The total phenol content of the dried teak leaf extract was 4.36% gallic acid equivalent (w/b), which was similar to the value reported by Budianto et al. (2023) for teak leaves ($4.3 \pm 0.15\%$ w/b). The phenol content in the teak leaf extract was also higher than that reported for *Thymus capitatus*, a plant known to inhibit seed germination. Algandaby and El-Darier (2018) reported that *T. capitatus* contained 3842 mg/100 g phenolic compounds, equivalent to 3.8% (w/b). These results indicate that teak leaf extract contains phenolic compounds with potential bioherbicidal activity.

The pH and EC values of the teak leaf extract at various concentrations are shown in Figure 1. The pH of the teak leaf extracts increased with increasing concentration, indicating alkaline pH levels, with values in the range of 8-8.3. The EC value of the teak leaf extract followed a similar trend to the pH value, with higher EC values observed with higher teak leaf extract concentrations, with the highest value at 40% concentration, with 1506 μ S. The pH value affects the availability of nutrients in the media. The salt and ion content of the solution influences the pH concentration and EC, which in turn affect plant growth by modifying the osmotic pressure and enzyme activity in the cells (Santos et al., 2021).

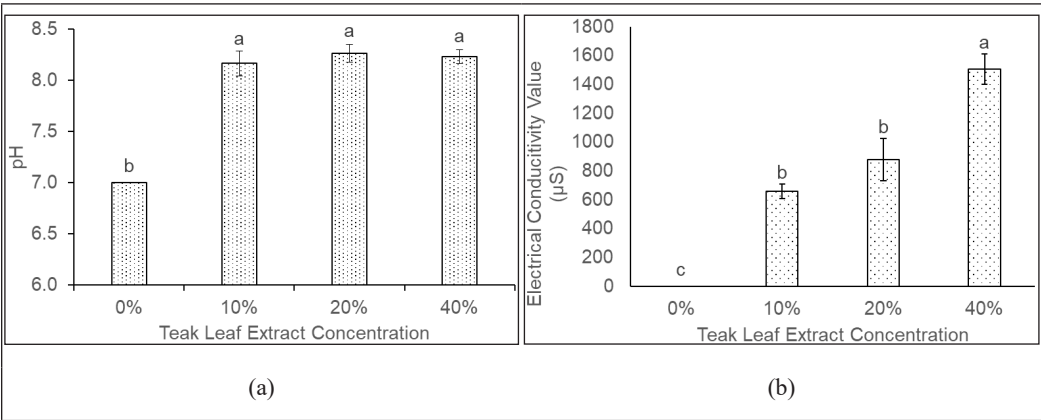


Figure 1. (a) pH value and (b) electrical conductivity value of teak leaf extract at various concentrations

Bioassays

In Figure 2a, the germination rate of seeds in the application of dried teak leaf extract concentration did not cause a significant decrease from the control treatment. Soybean resistance to teak leaf extract application at high pH and EC values is due to the soybean cultivar *Anjasmoro*.

Figure 2b shows no significant difference in the application of 10% and 20% teak leaf extract concentrations compared to the control for the soybean vigour index. Meanwhile, there was a significant difference in the vigour index of soybeans after application of the 40% concentration compared to the control, but no significant difference between the 40% and the 10% or 20% teak leaf extract concentrations. The decrease in soybean vigour index at higher extract concentrations suggests that elevated allelochemical exposure may interfere with early seedling development. Increasing extract concentration led to greater inhibition, resulting in a decrease in vigour index (Respatie et al., 2024).

This study found the highest abnormal germination rate in the 40% teak leaf extract treatment, at 16.67% (Figure 2c). The germination process requires amylase, which breaks down complex carbohydrates into simpler molecules (Gunathunga et al., 2024). Using a teak leaf extract containing phenol may inhibit amylase activity, resulting in reduced germination and abnormal sprouting. Allelochemicals inhibit microtubule formation, leading to abnormal growth (Reigosa et al., 2006).

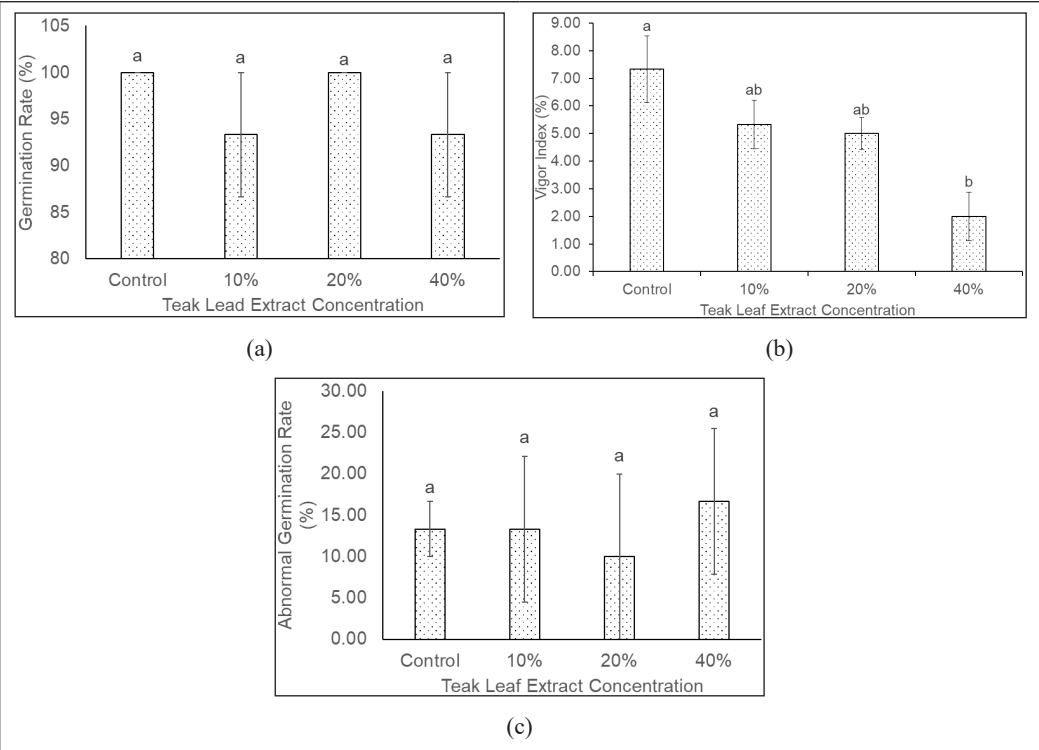


Figure 2. (a) Germination rate; (b) vigour index; and (c) incidence of abnormal soybean sprouts at different concentrations of teak leaf extract

The germination of *T. procumbens* weed showed significant differences (Figure 3a). In the control treatment, the germination rate of *T. procumbens* reached 80%, whereas applications of teak leaf extract at 10%, 20%, and 40% resulted in a 0% germination rate. The results of this study indicate that inhibition increases with increasing extract concentration, a finding similar to that reported by Respatie et al. (2024) for the Wedelia plant extract. Similar inhibitory effects of plant-based bioherbicides on weed germination have also been reported in several allelopathic plant species. Aqueous extracts of *Bidens bipinnata* L., *Ricinus communis* L., and *Piper tuberculatum* Jacq. significantly inhibited the germination and seedling growth of *B. bipinnata* (Lopes et al., 2022).

Teak leaves contain several phytotoxic compounds, including phenolics and quinones, which may contribute to germination inhibition (Kato-Noguchi, 2021). Therefore, a 10% concentration can completely inhibit the growth of *Tridax procumbens* and is considered effective when applied in subsequent stages of this study. The inhibition of seed germination may be attributed to allelochemicals in the bioherbicidal extract, which penetrate the seeds, reduce gibberellin content and α -amylase activity, and simultaneously increase antioxidant enzyme activity (Li et al., 2024). Furthermore, Somala et al. (2024) report that allelochemicals penetrating seed tissues induce the accumulation of reactive oxygen species (ROS) and elevate malondialdehyde (MDA) levels. This process results in oxidative damage to cellular membranes and may inhibit seed germination.

The vigour index of *T. procumbens* weed sprouts showed significantly different results between the control treatment and the teak leaf extract at 10%, 20%, and 40% concentrations (Figure 3b). In this case, applying a 10% concentration of dried teak leaf extract can inhibit the germination of *T. procumbens* weed seeds. Phenolic allelochemicals may interfere with seed imbibition and reduce α -amylase activity, thereby limiting carbohydrate mobilisation during germination (Madane & Patil, 2017). This disruption may subsequently affect mitotic activity and inhibit seed germination (Reigosa et al., 2006).

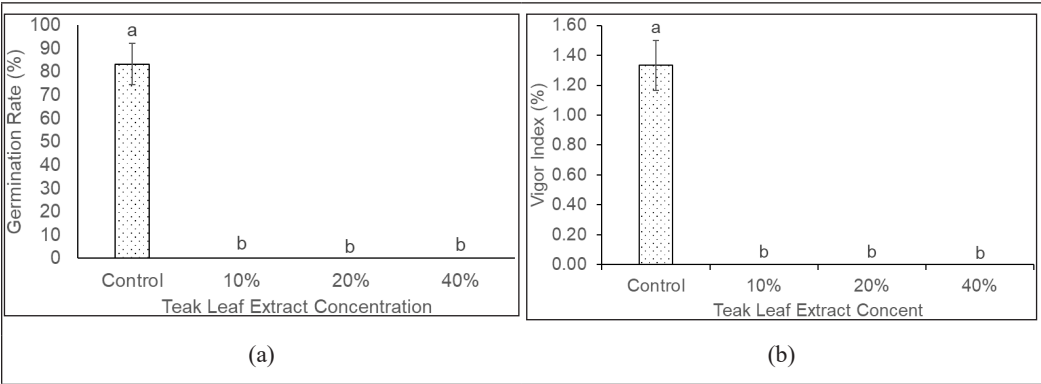


Figure 3. (a) Germination rate and (b) vigour index of *Tridax procumbens* at different concentrations of teak leaf extract

The 10% concentration was considered the most effective treatment because it achieved the same inhibitory effect on *T. procumbens* as the 20% and 40% concentrations, while requiring less raw material. In addition, 10% concentration did not negatively affect soybean germination. Therefore, 10% concentration was chosen for the greenhouse experiment.

Effect of Teak Leaf Extract Application on Soybean Plant Growth

The effect of the teak leaf extract application on soybean was indicated by the germination rate and root/shoot ratio at various teak leaf extract application times at 5 weeks after planting, as shown in Table 1. The germination percentage of soybean was similar between the control and teak leaf extract treatments, indicating that the application of 10% teak leaf extract did not affect soybean germination. At 5 weeks after planting, the control treatment showed the lowest root dry weight and the highest shoot dry weight, whereas the teak leaf extract treatments increased root dry weight and reduced shoot dry weight. These changes resulted in a higher root/shoot ratio, suggesting that soybean growth was more directed toward root development than shoot growth following teak leaf extract application. The high root/shoot ratio indicates that plant growth was more dominant in the roots compared to the shoots (Bláha, 2019). Similar growth responses have been reported in other allelopathic studies, where applying the allelopathic extract altered biomass allocation and root/shoot development (Xiao et al., 2024). One factor influencing this is the salinity resulting from the application of the teak leaf extract. The high EC values reflect the high salt concentrations in the teak leaf extracts (Corwin & Yemoto, 2017). High salt content can inhibit the initial growth of soybeans (Umida Toshtemirovna et al., 2023), which can lead to oxidative stress in soybeans, triggering lipid peroxidation and causing soybean growth inhibition, as shown by the root/shoot ratio (Uyun et al., 2024).

Table 1
The germination rate of soybean growth and the root/shoot ratio of soybean five weeks after planting at various teak leaf extract application times

Treatments	Germination Rate (%)	Root/Shoot Ratio
Control	81.67±20.64 a	0.30±0.05 c
D-7	83.33±12.77 a	0.37±0.10 bc
D-0	83.33±11.55 a	0.52±0.07 a
D+7	85.00±17.53 a	0.43±0.07 ab
CV (%)	20.65	17.87

Note. Numbers in the same column followed by the same character were not significantly different based on the Tukey Test ($\alpha = 5\%$)

The effect of teak leaf extract treatment at various application times on the germination rate and dry weight of *T. procumbens* weed at 5 weeks after planting is shown in Table 2. There were no weeds growing in the D-0 treatment. Allelopathic compounds, especially phenolic compounds, can affect plant germination and growth by inhibiting respiration and enzyme activity during germination and growth (Zohaib et al., 2016). Therefore, food reserves, water imbibition, and transportation were effectively inhibited during the D-0 application or planting time. In the D-7 application treatment, or 7 days before planting, there was also inhibition, although this was not complete inhibition. Dried teak leaf extract contains allelochemical compounds in the form of phenols, which can inhibit plant germination (Li et al., 2021). In addition, these compounds may inhibit the vigour or strength of plant growth. Allelochemicals such as phenols can inhibit enzyme activity by degrading the seed’s food reserves for germination. In particular, alpha-amylase activity decreased, thereby affecting the seeds’ viability (Padilha et al., 2022).

Table 2 shows that the weed dry weight decreased after teak leaf extract applications at D-0 and D+7. This is due to the application of teak leaf extract, which contains allelopathy and triggers oxidative stress, causing a decrease in the weed's dry weight (Uyun et al., 2024). Meanwhile, there was an increase in the weed dry weight because the inhibition of teak leaf extract only lasted for a few days at the beginning of the application at D-7. Therefore, the allelopathic effect of the teak leaf extract did not affect the weed dry weight in the D-7 application.

Treatments of various application times showed different germination rates, as shown in Figure 4. The control treatment and D+7 application exhibited the same results, where the seeds began to germinate on the 3rd day. Meanwhile, the D-7 application treatment showed that the seeds began germinating on the 5th day. In the D-0 application, no *T. procumbens* seeds germinated. Germination inhibition was found in the D-7 treatments, and the inhibitory effect occurred until the 11th day after application. One weed seed germinated on the 4th or 11th day after applying the teak extract. Therefore, the teak leaf extract can inhibit up to the 11th day after application. However, further observations are required to evaluate the bioherbicidal activity of teak leaf extract.

Table 2
The germination rate and dry weight of Tridax procumbens weeds that grew up to five weeks after planting in various treatments of teak leaf extract application time

Treatments	Germination Rate (%)	Weed Dry Weight (g)
Control	28.75±8.54 a	0.16±0.07 ab
D-7	6.25±2.50 b	0.19±0.05 a
D-0	0.00±0.00 b	0.00±0.00 c
D+7	22.50±6.45 a	0.09±0.03 b
CV (%)	4.42	3.76

Note. Numbers in the same column followed by the same character were not significantly different based on the Tukey Test ($\alpha = 5\%$)

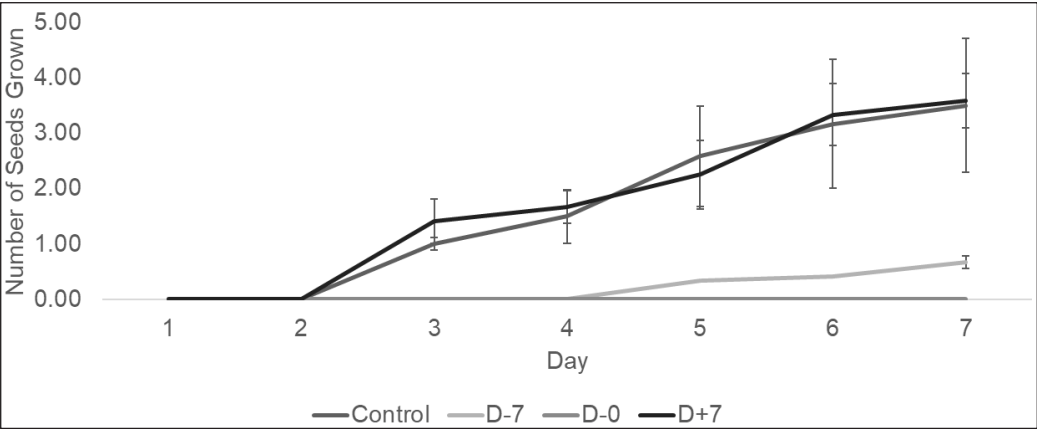


Figure 4. Germination rate of *Tridax procumbens* weed seeds at various teak leaf extract application times

The results of this study indicate that teak leaf extract at a concentration of 10% applied at planting time has the potential to be an environmentally friendly bioherbicide, as it inhibits the germination and growth of *T. procumbens* seedlings without disrupting soybean germination and growth. Furthermore, several regions in Indonesia have an abundance of underutilised fallen teak leaves. However, because this study was conducted in controlled environmental conditions (laboratory and greenhouse), further field-scale studies are needed to evaluate the effectiveness and stability of teak leaf extract under natural environmental conditions.

CONCLUSION

The 10% teak leaf extract treatment showed potential for effective inhibition of *T. procumbens* germination in soybean production under controlled conditions. In addition, application at planting time appeared to be the most effective timing for suppressing *T. procumbens* growth using teak leaf extract as a potential bioherbicide. However, as this study was conducted under controlled environmental conditions in the laboratory and greenhouse, additional field-scale research is required to assess the effectiveness and stability of teak leaf extract in natural environments.

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REFERENCES

- Algandaby, M. M., & El-Darier, S. M. (2018). Management of the noxious weed; *Medicago polymorpha* L. via allelopathy of some medicinal plants from Taif region, Saudi Arabia. *Saudi Journal of Biological Sciences*, 25(7), 1339-1347. <https://doi.org/10.1016/j.sjbs.2016.02.013>
- Bláha, L. (2019). Importance of root-shoot ratio for crops production. *Agronomy & Agricultural Science*, 2(2), 1-7. <https://doi.org/10.24966/AAS-8292/100012>
- Budianto, P., Suroto, S., Wasita, B., & Mirawati, D. K. (2023). *Tectona grandis* leaves: Determination of total flavonoid content, phenolic content, characterisation of the leaves, and compound identification in GC-MS. *Pharmacognosy Journal*, 15(1), 165-170. <https://doi.org/10.5530/pj.2023.15.24>
- Corwin, D. L., & Yemoto, K. (2017). Salinity: Electrical conductivity and total dissolved solids. *Methods of Soil Analysis*, 2(1), 1-17. <https://doi.org/10.2136/msa2015.0039>
- Gunathunga, C., Senanayake, S., Jayasinghe, M. A., Brennan, C. S., Truong, T., Marapana, U., & Chandrapala, J. (2024). Germination effects on nutritional quality: A comprehensive review of selected cereals and pulses changes. *Journal of Food Composition and Analysis*, 128, Article 106024. <https://doi.org/10.1016/j.jfca.2024.106024>
- Kato-Noguchi, H. (2021). Phytotoxic substances involved in teak allelopathy and agroforestry. *Applied Sciences*, 11(8), Article 3314. <https://doi.org/10.3390/app11083314>
- Kaur, S., Kaur, R., & Chauhan, B. S. (2018). Understanding crop-weed-fertiliser-water interactions and their implications for weed management in agricultural systems. *Crop Protectio*, 103, 65-72. <https://doi.org/10.1016/j.cropro.2017.09.011>
- Kole, R. K., Paul, P., Saha, S. D., & Mukhopadhyay, S.K. (2016). Chemistry and bio-efficacy of teak leaf for weed control in wheat. *Allelopathy Journal*, 39(2), 191-2014. <https://www.researchgate.net/publication/324694829>
- Li, B., Wu, W., Shen, W., Xiong, F., & Wang, K. (2024). Allelochemicals released from rice straw inhibit wheat seed germination and seedling growth. *Agronomy*, 14(10), Article 2376. <https://doi.org/10.3390/agronomy14102376>
- Li, J., Chen, L., Chen, Q., Miao, Y., Peng, Z., Huang, B., Guo, L., Liu, D., & Du, H. (2021). Allelopathic effect of *Artemisia argyi* on the germination and growth of various weeds. *Scientific Reports*, 11(1), Article 4345. <https://doi.org/10.1038/s41598-021-83752-6>
- Lopes, R. W. N., Marques Morais, E., Lacerda, J. J. de J., & Araújo, F. D. da S. (2022). Bioherbicidal potential of plant species with allelopathic effects on the weed *Bidens bipinnata* L. *Scientific Reports*, 12(1), Article 12423. <https://doi.org/10.1038/s41598-022-16203-5>
- Madane, A. N., & Patil, B. J. (2017). Allelopathic effect of *Eupatorium odoratum* L. on amylase activity during seed germination of *Cicer arietinum* L. and *Cajanus cajan* (L) Millsp. *Bioscience Discovery*, 8(1), 82-86.
- Motmainna, M., Juraimi, A. S., Hasan, M., Asib, N. B., Islam, A. K. M. M., & Ahmad-Hamdani, M. S. (2024). Identification of phytochemicals in *Cleome ruidosperma* DC. methanol extract and evaluate its efficacy on some common rice field weeds. *Pertanika Journal of Tropical Agricultural Science*, 47(1), 213-232. <https://doi.org/10.47836/pjtas.47.1.16>
- Padilha, M. S., Coelho, C. M. M., & Ehrhardt-Brocardo, N. C. M. (2022). Viability and performance of wheat seedlings after artificial seed ageing. *Journal of Seed Science*, 44, Article e202244037. <https://doi.org/10.1590/2317-1545v44261925>

- Rajak, P., Roy, S., Ganguly, A., Mandi, M., Dutta, A., Das, K., Nanda, S., Ghanty, S., & Biswas, G. (2023). Agricultural pesticides - friends or foes to biosphere? *Journal of Hazardous Materials Advances*, 10, Article 100264. <https://doi.org/10.1016/j.hazadv.2023.100264>
- Reigosa, M. J., Pedrol, N., & González, L. (Eds.). (2006). Allelopathy: A physiological process with ecological implications. Springer. <https://doi.org/10.1007/1-4020-4280-9>
- Respatie, D. W., Purwantoro, A., Indradewa, D., Uyun, Q., & Hafidhotul Ilmiah, H. (2024). Evaluation of *Wedelia* water extract on soybean, purple nutsedge, and billygoat seeds germination. *Jurnal Agronomi Indonesia (Indonesian Journal of Agronomy)*, 52(2), 185-210. <https://doi.org/10.24831/jai.v52i2.56918>
- Rodrigues dos Reis, A., & Vivian, R. (2011). Weed competition in the soybean crop management in Brazil. In T.-B. Ng (Ed.), *Soybean - Applications and Technology* (pp. 67-88). InTech. <https://doi.org/10.5772/15775>
- Saludes-Zanfaño, M. I., González-Hernández, A. I., Vivar-Quintana, A. M., & Morales-Corts, M. R. (2024). Phytotoxicity of phenolic compounds of *Pistacia vera* leaves and its potential use as bioherbicide. *Crop Protection*, 184, Article 106812. <https://doi.org/10.1016/j.cropro.2024.106812>
- Santos, C., Fonseca, J., Coutinho, J., Trindade, H., & Jensen, L. S. (2021). Chemical properties of agro-waste compost affect greenhouse gas emission from soils through changed C and N mineralisation. *Biology and Fertility of Soils*, 57(6), 781-792. <https://doi.org/10.1007/s00374-021-01560-6>
- Scavo, A., Pandino, G., Restuccia, A., & Mauromicale, G. (2020). Leaf extracts of cultivated cardoon as potential bioherbicide. *Scientia Horticulturae*, 261, Article 109024. <https://doi.org/10.1016/j.scienta.2019.109024>
- Silva, A. A. P., Oliveira Neto, A. M., Guerra, N., Helvig, E. O., & Maciel, C. D. G. (2015). Períodos de Interferência entre Plantas Daninhas e a Cultura da Soja RR® na Região Centro Ocidental Paranaense [Interference Periods Among Weeds and Soybean RR™ Crops in the Western Center Area of the Brazilian State of Paraná]. *Planta Daninha*, 33(4), 707-716. <https://doi.org/10.1590/S0100-83582015000400009>
- Somala, N., Manichart, N., Laosinwattana, C., Wichittrakarn, P., Yoneyama, K., Teerarak, M., & Chotsaeng, N. (2024). Oxidative damage in *Echinochloa crus-galli* seeds exposed to *Diaporthe* sp. (Diaporthales, Ascomycota) fungal extract during germination. *Frontiers in Agronomy*, 6, Article 1456168. <https://doi.org/10.3389/fagro.2024.1456168>
- Umida Toshtemirovna, N., Kamal Kizi, K. N., & Zavkiddinova, A. S. (2023). The effect of salinity on the initial growth of soy varieties. *International Journal of Current Science Research and Review*, 6(3), 1957-1962. <https://doi.org/10.47191/ijcsrr/V6-i3-08>
- Uyun, Q., Respatie, D. W., & Indradewa, D. (2024). Unveiling the allelopathic potential of *Wedelia* leaf extract as a bioherbicide against purple nutsedge: A promising strategy for sustainable weed management. *Sustainability*, 16(2), Article 479. <https://doi.org/10.3390/su16020479>
- Xiao, Y., Tan, J., Yu, Y., Dong, J., Cao, L., Yao, L., Zhang, Y., & Yan, Z. (2024). Phytotoxic effects and potential allelochemicals from water extracts of *Paulownia tomentosa* flower litter. *Agronomy*, 14(2), Article 367. <https://doi.org/10.3390/agronomy14020367>
- Zohaib, A., Abbas, T., & Tabassum, T. (2016). Weeds cause losses in field crops through allelopathy. *Notulae Scientiae Biologicae*, 8(1), 47-56. <https://doi.org/10.15835/nsb.8.1.9752>

Field Performance of Hybrid Corn Varieties (*Zea mays*) Applied with Gibberellic Acid 3 (GA 3) at Cabagan, Isabela, Philippines

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ABSTRACT

Corn (*Zea mays*) world's most productive crop, serving multiple uses in livestock feed, biofuel, and ethanol production industries. The study aimed to evaluate the performance of hybrid corn varieties applied with Gibberellic Acid 3 (GA 3). It was employed in Randomised Complete Block Design (RCBD) with a 4 × 2 factorial arrangement at eight treatment combinations. Factor A consisted of four different GA3 concentrations: Without GA 3 (WOG), 175 ml of GA 3 per hectare (175GA3),

200 ml of GA 3 per hectare (200GA3), and 225 ml of GA3 per hectare (225GA3). Factor B included two corn varieties: NK8840 (NK8) and NK6410 (NK6). Data collection focused on growth and yield parameters for each treatment combination, analysed using two-way ANOVA to assess the interaction of GA3 concentration and corn variety. Treatment differences were determined by Tukey's HSD test, with P-values ($P < 0.05$) obtained for plant height (PH), leaf count (LC), ear length (LE), ear diameter (ED), biomass yield (BY), weight of kernels (WK), and computed yield (CY). Results indicated that GA3 significantly affected BY and CY, with the 200 ml GA3 treatment yielding better results than other concentrations. While specific parameters did not show statistical significance, NK6 outperformed NK8 in terms of BY and CY.

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The application of 200 ml GA3 resulted in the highest recorded yield and enhanced crop performance, suggesting it as a viable strategy for optimising corn production and improving farmers' economic returns.

Keywords: Ear, gibberellic acid 3, growth, hybrid corn, performance, tassel

INTRODUCTION

Hybrid corn (*Zea mays*) is recognised as one of the most important staple food crops globally, particularly in the Philippines. It serves as a primary source of food, feed, and industrial raw materials (Rustia et al., 2022; Solaimalai et al., 2020; Shiferaw et al., 2011). Despite its significance, low productivity remains a persistent challenge for farmers and the agricultural sector (Giller et al., 2021; Shiferaw et al., 2011). According to the Department of Agriculture Regional Field Office III, approximately 600,000 farm households depend on corn as their main source of income. Isabela and Bukidnon are the leading producers of corn in the Philippines, contributing 1.8 million metric tons to the national output (Philippine Competition Commission [PCC], 2021). Gibberellic acid (GA3) is a plant growth regulator that presents a promising solution to overcoming productivity challenges and improving corn yields. Camara et al. (2018) reported that GA3 significantly enhances plant growth and development while being economically viable for production. Isabela has been recognised as the top corn-producing province, contributing between 9% and 16% of the country's overall corn output (Ocampo et al., 2015; Reyes et al., 2009). However, recent projections indicate a substantial decline in corn production. The Philippine Statistics Authority (PSA) (2024) reports that standing crop estimates from April to June 2024 have decreased by 19.1% compared to 2023, dropping from 1.47 million metric tons to 1.19 million metric tons. This decline is attributed to adverse weather conditions, including dry spells early in the year and severe flooding in September and December, both of which significantly impacted crop yields. As a result, Isabela has fallen to the second position nationally in corn production.

To address these challenges and enhance corn productivity, there is growing interest in plant growth regulators (PGRs), particularly gibberellic acid (GA3). GA3 is an essential plant hormone known for stimulating stem elongation, leaf expansion, and overall plant vigour (Al-Shaheen et al., 2018; Gao et al., 2020). Numerous studies confirm that applying GA3 enhances the growth and yield of corn across various environmental conditions. However, its effectiveness depends on several factors, such as planting density. Optimising plant spacing is crucial for improving light interception, nutrient availability, and creating a favourable microclimate, ensuring that each plant has access to the necessary resources to benefit fully from GA3 (Anjum et al., 2017; Camara et al., 2018; Majender et al., 2015). Overcrowding can intensify competition among plants, hindering their ability to utilise

GA3 effectively. Conversely, appropriate spacing can substantially enhance productivity (Noda-Leyva & Martín-Martín, 2017).

On the other hand, despite the rising popularity of GA3, further research is needed to understand its interaction with various planting distances, particularly in soil conditions like those found in clay-dominated fields. Additionally, GA3 has the potential to enhance crop performance under abiotic stress. For example, the external application of GA3 has been shown to improve corn growth under salinity stress by increasing chlorophyll content, reducing sodium uptake, and enhancing potassium uptake, ultimately leading to improved photosynthetic activity and greater resilience (Mukkarram et al., 2021; Shadzad et al., 2021). Further investigation into the combined effects of GA3 and foliar fertiliser applications revealed elevated antioxidant enzyme activities, reduced oxidative damage, and improved nutrient uptake, highlighting its potential as a protective agent in adverse conditions (El-Nwehy & Afify, 2023). Similarly, a study on GA3 and mepiquat chloride (MC) found that a foliar fertiliser with 100 ppm GA3 resulted in approximately a 33% increase in corn yield compared to the control, demonstrating its role in reducing stress-induced yield losses.

GA3 promotes and encourages cell elongation and division, which ultimately boosts overall yield (Ayyub et al., 2013; Zaman et al., 2015). Findings indicate that the application of GA3 can positively influence crop productivity under various irrigation conditions. Significantly, when applied during optimal irrigation periods, GA3 has shown the potential to yield the highest crop output. Importantly, even under drought stress, the application of GA3 has proven effective in enhancing crop resilience and achieving higher yields under ideal irrigation situations (Sawar et al., 2017). This capability underscores GA3's significant role as an important agricultural strategy, enhancing productivity under typical conditions and strengthening corn's ability to withstand drought. Furthermore, it has been demonstrated that GA3 improves seed germination and growth of corn in saline conditions, indicating its ability to help crops endure environmental stresses (Ge et al., 2023; Rauf et al., 2022).

Hybrid corn varieties have become the preferred choice for farmers due to their remarkable qualities, such as resistance to insect pests and diseases, and their adaptability to environmental conditions. While hybrid corn has played a leading role in advancing agricultural technology, its development and widespread adoption have also inadvertently contributed to current challenges within the corn farming sector (Ramey, 2010).

Current research advances provide field-based, site-specific evidence on how GA3 concentrations influence the productivity of hybrid corn varieties under real agricultural conditions. In contrast to previous studies that focused on single varieties, controlled environments, or stress simulations, this research aims to identify variety characteristics and optimal GA3 applications that improve performance amidst climate disruptions. This study aims to make the GA3 application more practical, cost-effective, and applicable for

farmers, improving our understanding of its role in corn growth. The anticipated outcomes will offer valuable insights into GA3 as a plant growth regulator, helping farmers determine the optimal concentration for their specific hybrid corn varieties.

METHODOLOGY

Study Area

The study was conducted at the Experimental area of Isabela State University, Cabagan Campus, located in Cabagan, Isabela, Philippines. This location, found at a torrid zone with a 17.4144° N latitude and 121.7670° E longitude, experiences high temperatures, which is characteristic of the Philippines. The experiment was carried out over the dry season from March 2023 to July 2023. This month, prolonged heat, low humidity, or limited rainfall could have amplified or suppressed GA 3 effects, so the observed growth and yield may differ under wet or cooler seasons.

Treatments, Experimental Design, and Soil Sampling

The field trials covered 341 square meters, divided into three blocks, each subdivided into 8 equal plots measuring 3 m × 3 m with 1.0 m alleyways between blocks and plots. Soil samples were collected at a depth of 6-8 inches, with composite samples representing the entire area. Following debris, weed, and topsoil removal, samples were collected using a shovel, bolo, and post hole digger, then transported in pails. The samples were pulverised and air-dried for 7 days. Uniform 1-kilogram samples were obtained using a quartering technique and sent to the Department of Agriculture-Soil Laboratory-Cagayan Valley Research Centre (DA-SL-CVRC) in San Felipe, Ilagan, Isabela, for analysis. Based on the soil analysis recommendations, detailed in Table 1, both inorganic and organic fertilisers were applied. Organic fertilisers, specifically carbonised rice hull and vermicompost, were administered at 450 grams per plot.

Table 1
Mode of application, type and rate of fertiliser applied in the study

Mode of Application	Type of Fertiliser	Rate of Application
Basal	Organic Fertiliser (Carbonised Rice Hull and Vermicompost)	450 g per plot
	14-14-14 (Complete Fertiliser)	256.95 g per plot
Side Dressing	46-0-0 (Urea)	58.5 g per plot
	0-0-60 (Muriate of Potash)	31.5 g per plot

Note. This table is the result of soil analysis of the Department of Agriculture-Soil Laboratory-Cagayan Valley Research Centre (DA-SL-CVRC) in San Felipe, Ilagan, Isabela

Inorganic fertilisers, including Urea (46-0-0), Muriate of Potash (0-0-60), and Complete Fertiliser (14-14-14), were applied at rates of 58.5 grams, 31.5 grams, and 256.95 grams, respectively.

The experiment was structured as a 4×2 randomised complete block design (RCBD) using a factorial approach. Factor A evaluated the effects of different durations of drought and flood stress, consisting of the following treatments: A1 - Without GA3 (WOG), A2 - 175 ml of GA3 per hectare (175GA3), A3 - 200 ml of GA3 per hectare (200GA3), and A4 - 225 ml of GA3 per hectare (225GA3). The selected GA3 concentrations were chosen because they are within agronomically proven, safe, and cost-effective ranges that enhance corn growth without causing abnormal elongation or yield reduction. These concentrations were intended to capture the most practical and effective GA3 response under field conditions, ensuring both biological effectiveness and applicability for farmers. The control group, designated as A1 in the experiment, served as a baseline reference point against which the other treatment groups were compared. A1 - Control refers to the plants that were not treated with GA3. The purpose of the control group was to provide a standard for comparison, enabling an accurate assessment of the effects of the applied treatments.

On the other hand, Factor B represented the different hybrid varieties of corn used in the study. The first variety, NK8840 (NK8), matures in 115-120 days after planting (DAP) and has a yield potential of 12 metric tons per hectare (MT/ha). This variety is well adapted to the Isabela, Cagayan Valley Region, particularly in Upper Vega, Ilocos Region, and Central Luzon. It is noted for its better drought resistance and ease of dehusking and harvesting. The second variety, NK6410 (NK6), matures in 100-105 DAP and also has a potential yield of 12 MT/ha. NK6410 is suitable for a wider range of regions, including Lower Vega in the Cagayan Valley, Ilocos Region, Central Luzon, South Luzon, Bicol Region, and Mindanao Low Elevation. This variety is recognised for producing high-quality grain and its desirable field performance, making it easy to shuck and harvest. To maintain control of the experiment and enable a thorough study, the selection focused exclusively on these two hybrid varieties.

Planting, Planting Materials, and Crop Management

The materials needed to implement the study were procured from an Agricultural Supply store in Ugad, Cabagan, Isabela, such as hybrid corn seeds, fertilisers like Complete, Urea, and Muriate of Potash and insecticide, while GA 3 was obtained in authorized online retailer. Other items, including vermicast, wheelbarrow, hand tractor, placards, gardening tools, pegs, meter stick, and sprayer, were borrowed from the school nursery.

The land preparation operation started with ploughing at a double-pass using a four-wheeled tractor, followed by harrowing one week prior to planting. Any foreign materials and weeds were removed. The research areas were then divided into three columns and eight rows, measuring 3×3 meters per plot. The soil surface was flattened using a rake before planting started.

In furrows, Hybrid corn seeds were sown at a depth of 2 to 5 cm, a total of 130 seeds per plot accommodating 10 rows, with 13 hills per row each subplot, To assure the high germination two seeds per hill was planted while it maintained the spacing of 20 cm between hills and 25 cm between rows, However during the conduct of study, poor germination was observed, particularly in NK6410 variety, replanting at 7 and 15 days after sowing (DAS) was conducted. This was performed by filling any vacant hills to ensure the uniform density across all the plots, an important factor for reliably evaluating the effects of the GA 3 treatments. Patchy plant stands can lead to uneven competition for the resources such as light, water and nutrients. By resolving this problem early in the growth cycle, it should maintain the plant populations, ensuring that any evident differences in productivity could be credited to the treatments rather than variations in plant establishment.

The GA 3 was diluted in 115.2 ml of water and applied in a fine foliar spray using a knapsack sprayer, maintaining constant spray volume and pressure, to ensure equal absorption of GA 3 across all plots. This was carried out at 15, 30, and 45 days after sowing (DAS). Applications sustained until the leaves were uniformly wet. All plots obtained corresponding concentrations, volume of spray, and application interval to lessen variability in absorption. High temperature was considered in the potential for GA 3 degradation and applications, it was sprayed during early morning hours when temperatures are lower, and its evaporation rates are minimal.

A submersible pump was used in irrigation management and was operated every 5 days. Weed management was performed through manual removal using a hand trowel. Initial management of fall armyworm infestations involved hand-picking the pests. Harvesting of mature corn cobs was manually collected, followed by manual threshing by removing the husks and separation of kernels. Lastly, kernels were sun-dried until they reached 12-14% of moisture content.

General Observation and Collection of Data

The several key growth and yield parameters in data collection focus on the following; first is the measuring of plant height (PH) using meter stick from the base of the stem to the tip of the tallest leaf, this was taken 45 DAS, which also coincided with the final GA 3 application, chosen to capture the peak response to the treatment and provide a practical yet reliable indicator of its effect. Leaf count (LC) was recorded following the GA 3 application.

For corn yield, the length of ears (LE) is measured from the base of the plant to the tip of the cob after dehusking. The calliper ear diameter (ED) was assessed, while the computed yield (CY) was calculated by weighing the harvested marketable dry kernels for each treatment. Its weight was converted into per-hectare yield to reflect the actual field performance, and the weight of 1,000 kernels (WK) was determined by drying its samples.

Analysis of Data

To analyse the effect of different concentrations of GA3 and various corn varieties on productivity parameters, a statistical analysis called Analysis of Variance (ANOVA) was conducted. Prior to the analysis, several assumptions underlying the ANOVA model were carefully checked. These assumptions included the normality of the residuals, homogeneity of variances across groups, and independence of individual observations.

The Shapiro-Wilk test was used to assess the normality of the residuals, while Levene's test was employed to evaluate the consistency of variances. The appropriate data transformations were implemented to normalise the data and stabilise variances, thus ensuring the validity and reliability of the statistical assumptions drawn from the ANOVA, while Tukey's Honestly Significant Difference (HSD) test was utilised to determine the significance of the differences observed among the treatment means. The results were carried in terms of the mean values together with the standard deviation (SD), and lastly, the significance level of $P < 0.05$ to ascertain the statistical significance of the findings was also established.

RESULTS AND DISCUSSION

Plant Height

The data on plant height under various concentrations of gibberellic acid (GA3) and different corn varieties (Table 2), despite not showing statistically significant differences in results, offers insightful observations with practical implications. The treatment with 200 ml of GA3 per hectare (200GA3) achieved the highest mean height of 147.67 ± 6.79 cm, suggesting a potential positive influence of GA3 at this concentration. This was followed by the treatment without GA3 (WOG), which had a mean height of 144.67 ± 3.79 cm, and the treatment with 175 ml of GA3 per hectare (175GA3), which had a mean height of 136.17 ± 4.71 cm. The lowest mean height was observed in the treatment with 225 ml of GA3 per hectare (225GA3), with a mean of 135.00 ± 5.88 cm. However, the results did not identify these differences as statistically significant.

As shown in Table 3, the corn variety NK6 (NK6410) had a mean plant height (PH) of 143.67 ± 2.79 cm, while NK8 (NK8840) had a mean PH of 138.08 ± 2.79 cm. Despite this numerical difference, it was not statistically significant, suggesting that the height variation between these two varieties is minimal and likely due to random variation rather than a true difference in growth characteristics. Both NK6410 and NK8840 are hybrid corn varieties developed by Syngenta Philippines, known for their high yield potentials and adaptability to various regions. NK6410 matures in 100-105 days and has a potential yield of 12 metric tons per hectare.

Table 2
Effects of plant height, leaf count, ear length, ear diameter, biomass yield, and computed yield in different concentrations of GA3 in hybrid corn

Factor A	PH (cm)	LC	LE (cm)	ED (mm)	BY (g)	WK (g)	CY (kg)
WOG	144.67 ± 3.79	9.00 ± 0.29	12.50 ± 0.08	37.17 ± 0.545	1748.33 ± 109.25 ^b	283.00 ± 6.21	1673.67 ± 466.13 ^c
175GA3	136.17 ± 4.71	8.17 ± 0.54	12.67 ± 0.08	35.83 ± 0.795	1735.00 ± 122.58 ^b	270.83 ± 5.97	1907.67 ± 232.13 ^c
200GA3	147.67 ± 6.79	9.17 ± 0.46	13.00 ± 0.42	37.17 ± 0.545	1986.67 ± 128.42 ^a	276.83 ± 0.04	2600.67 ± 460.88 ^a
225GA3	135.00 ± 5.88	8.50 ± 0.21	12.17 ± 0.42	36.33 ± 0.295	1961.00 ± 103.42 ^a	276.50 ± 0.29	2377.17 ± 237.38 ^b

Note. WOG = Without GA3; 175GA3 = 175 ml of GA3 per hectare; 200GA3 = 200 ml of GA3 per hectare; 225GA3= 225 ml of GA3 per hectare
*PH = plant height; LC = leaf count; LE = length of ears; ED = ear diameter; BY = biomass yield; WK = Weight of 1000 kernels; CY = computed yield;
cm = centimetre; mm = millimetre; g = gram and kg = kilogram. The different factors are presented along with their respective standard deviations (SD) using
the format mean ± standard deviation. Significant differences at a 5% probability level (P < 0.05) were tested

It is adaptable to regions like Cagayan Valley, Ilocos Region, Central Luzon, South Luzon, Bicol Region, and Mindanao's low elevations. NK8840 matures in 115-120 days, also with a potential yield of 12 metric tons per hectare, and is suitable for Cagayan Valley (Upper Vega), Ilocos Region, and Central Luzon. The slightly shorter heights of NK6410 and NK8840 could be attributed to their hybrid nature, potentially bred for specific traits like drought resistance or suitability for certain climates, which might result in shorter stature without compromising yield.

Furthermore, Table 4 illustrates the interaction between GA3 concentrations and corn varieties, showing that the combination of 200GA3 and NK6 resulted in the tallest plants at 148.90 ± 8.03 cm. Despite the lack of statistical significance, these findings indicate a trend where 200GA3 could be considered optimal for enhancing corn plant height. Practical applications of this study suggest that using 200 ml/ha of GA3 could potentially maximise plant height, contributing to better canopy structure and potentially higher yield. However, challenges such as fall armyworm infestations, which hindered plant growth during the first 30 days after planting (DAP), adversely affecting plant growth, must be managed to realise the full benefits of GA3 treatments. This study underscores the importance of integrated pest management alongside growth regulator applications to optimise crop performance. Abrahams et al. (2017) observed that infestations of fall armyworm can cause growth distortion, reduced crop yield, and in severe cases, total crop failure.

Leaves Count

The study investigated how different concentrations of GA3 and various corn varieties influence leaf count. Although the observed trends were interesting, the differences between the treatments were not statistically significant. Table 2 shows that the treatment with 200 ml/ha of GA3 (200GA3) had the highest average leaf count at 9.17 ± 0.46 , while the treatment with 175 ml/ha of GA3 (175GA3) recorded the lowest average at 8.17 ± 0.54 . Furthermore, neither the concentrations of GA3 nor the corn varieties significantly affected leaf count. A slight numerical difference was observed in leaf count between the NK6 and NK8 varieties, with means of 8.83 and 8.58, respectively, but this difference was not statistically significant. When examining the combination of both GA3 concentrations and corn varieties, no significant differences were found; however, leaf counts ranged from 7.63 to 9.27.

The combination of 200GA3 and NK6 produced the highest leaf count, followed by 225GA3 + NK6 with a mean of 8.77 ± 0.14 counts, and 200GA3 + NK8 with 8.87 ± 0.24 counts. Other combinations included WOG + NK8 (8.83 ± 0.20 counts), 175GA3 + NK8 (8.57 ± 0.06 counts), 175GA3 + NK6 (8.00 ± 0.63 counts), and 225GA3 + NK8 (7.63 ± 0.99 counts), with the latter being the lowest. Despite these differences, the results indicated that there were no significant differences among the treatments.

Length of Ears

The effect of ear length in relation to varying concentrations of GA3 and different corn varieties yielded nuanced results; however, the findings did not show statistically significant differences. Among the GA3 concentrations, the application of 200 ml/ha (200GA3) resulted in the highest mean ear length of 13.00 ± 0.42 cm, while the shortest ear length was noted in the 225 ml/ha treatment (225GA3), with an overall mean of 12.17 ± 0.42 cm. Additionally, the NK6 variety produced a higher mean ear length of 13.17 ± 0.59 cm, compared to the NK8 variety, which had a mean of only 12.00 ± 0.59 cm. The analysis of the interaction between the concentrations and varieties, as shown in Table 4, revealed that the combination of 200GA3 and the NK6 variety yielded the longest mean ear length of 13.73 ± 1.15 cm. However, there were no significant differences in ear length among the evaluated treatments.

Ear Diameter

In ear diameter (ED) showed in the study that while different concentrations GA 3 and various corn varieties did not produce statistically significant differences, intriguing insights emerged. Table 2 indicates that the 200 ml/ha GA 3 treatment and the control (WOG) group exhibited the highest ear diameters, with the 175 ml/ha GA 3 treatment showing slightly reduced values, suggesting that GA 3 application may not significantly impact ED but offers flexibility in maintaining it.

A more detailed analysis presented in Table 3 indicated that the NK6 variety had a marginally larger mean ear diameter of 37.33 ± 0.71 mm, compared to NK8, which had a mean ear diameter of only 35.92 ± 0.71 mm. This difference was not statistically significant, highlighting the overall consistency of ED across these varieties. The interaction effects shown in Table 4 further reinforced this consistency, revealing variations in mean values across combined treatments but no significant impact on ED. Ultimately, although no statistically significant differences were detected, the findings emphasise practical flexibility in managing crop yield and minimising variability by considering GA3 applications and the choice of corn varieties.

Biomass Yield

The findings on biomass yield (BY) showed significant variations influenced by both GA 3 concentrations and corn varieties. As presented in Table 2, it was revealed that the 200GA3 treatment yielded the highest average BY with a mean of 1986.67 ± 128.42 g, and statistical analysis confirmed significant differences among GA 3 levels, particularly between the 175 ml/ha treatment and the control (WOG), underscoring the role of GA 3 in yield trends, the same with the research showing GA 3 can enhance biomass yield in crops (Singh & Singh, 2019).

Table 3
Response of two varieties of hybrid corn in various parameters

Factor B	PH (cm)	LC	LE (cm)	ED (mm)	BY (g)	WK (g)	CY (kg)
NK8	138.08 ± 2.79	8.58 ± 0.14	12.00 ± 0.59	35.92 ± 0.71	1572.5 ± 217.92 ^b	272.50 ± 4.29	1840.00 ± 299.79 ^b
NK6	143.67 ± 2.79	8.83 ± 0.14	13.17 ± 0.59	37.33 ± 0.71	2008.33 ± 217.92 ^a	281.08 ± 4.29	2439.58 ± 299.79 ^a

Note. NK8 = NK8840 corn variety; NK6 = NK6640 corn variety
*PH = plant height; LC = leaf count; LE = length of ears; ED = ear diameter; BY = biomass yield; WK = Weight of 1000 kernels; CY = computed yield; cm = centimetre; mm = millimetre; g = gram and kg = kilogram. The different factors are presented along with their respective standard deviations (SD) using the format mean ± standard deviation. Significant differences at a 5% probability level (P < 0.05) were tested

Table 4
Interaction of effects of hybrid corn varieties applied with different concentrations of GA3

Treatments	PH (cm)	LC	LE (cm)	ED (mm)	WK (g)
WOG + NK8	142.50 ± 1.63	8.83 ± 0.20	11.63 ± 0.96	35.43 ± 0.38	286.00 ± 9.21
175GA3 + NK8	138.13 ± 2.74	8.57 ± 0.06	12.30 ± 0.29	35.69 ± 0.12	262.00 ± 14.79
200GA3 + NK8	146.70 ± 5.83	8.87 ± 0.24	12.40 ± 0.19	35.76 ± 0.05	266.00 ± 10.79
225GA3 + NK8	124.90 ± 15.97	7.63 ± 0.99	12.20 ± 0.39	35.53 ± 0.28	276.00 ± 0.79
WOG + NK6	146.47 ± 5.60	9.07 ± 0.44	12.97 ± 0.39	34.52 ± 1.29	280.00 ± 3.21
175GA3 + NK6	134.47 ± 6.40	8.00 ± 0.63	13.42 ± 0.84	36.12 ± 0.31	279.67 ± 2.88
200GA3 + NK6	148.90 ± 8.03	9.27 ± 0.64	13.73 ± 1.15	38.77 ± 2.96	287.67 ± 10.88
225GA3 + NK6	144.90 ± 4.03	8.77 ± 0.14	12.03 ± 0.56	34.68 ± 1.13	277.00 ± 0.21

Note. WOG = Without GA3; 175GA3 = 175 ml of GA3 per hectare; 200GA3 = 200 ml of GA3 per hectare; 225GA3= 225 ml of GA3 per hectare; NK8= NK8840 corn variety; NK6 = NK6640 corn variety
PH = plant height; LC = leaf count; LE = length of ears; ED- ear diameter; BY = biomass yield; WK = Weight of 1000 kernels; cm = centimeter; mm = millimeter; g = gram and kg = kilogram. The different factors are presented along with their respective standard deviations (SD) using the format mean ± standard deviation. Significant differences at a 5% probability level (P < 0.05) were tested

Table 3 further highlighted the importance of genetic factors, revealing significant differences in BY among corn varieties, with NK6 producing a higher mean BY with a final mean of 2008.33 ± 217.9 g than NK8, a finding echoed in other studies where cultivar choice significantly impacted yield components. The interaction analysis in Table 4 presented a more complex picture: the combination of 200 ml/ha GA 3 with the NK6 variety resulted in the highest BY (2596.67 ± 806.25 g), while the NK8 variety showed lower yields, especially when treated with no GA 3 (WOG at 1376.67 ± 413.75 g), reinforcing that the 200 ml/ha GA 3 concentration was particularly effective across both varieties tested.

Figure 1 illustrates the interaction analysis, demonstrating that combinations such as 200 ml/ha GA3 with NK6 achieved the highest mean BY of 2596.67 ± 806.25 g, followed by 200 ml/ha GA3 with NK8 at a mean of 1910.00 ± 119.58 g. Conversely, the combination of no GA3 with NK8 produced the lowest BY with a mean of 1376.67 ± 413.75 g. These experimental results indicate statistically significant differences, highlighting that the 200 ml/ha GA3 treatment performed well across the tested varieties. The interaction between plant growth regulators, like GA3, and crop varieties has been shown to significantly affect both yield and quality. For instance, a study on the effect of GA3 on the flowering and yield of hybrid rice parental lines found that applying GA3 at specific growth stages significantly increased plant height, internode length, and total dry biomass.

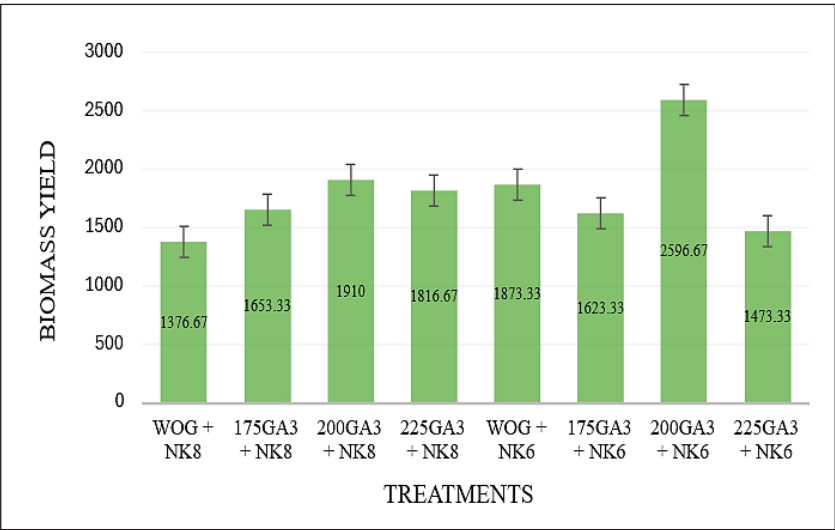


Figure 1. Interaction of effects of hybrid corn varieties applied with different concentrations of GA3 on biomass yield

Note. WOG = Without GA3; 175GA3 = 175 ml of GA3 per hectare; 200GA3 = 200 ml of GA3 per hectare; 225GA3 = 225 ml of GA3 per hectare; NK8 = NK8840; NK6 = NK6640

Weight of 1000 kernels

As observed in Table 2, the weight of 1000 kernels (WK) was influenced by various concentrations of GA3. Notably, the treatment without GA3 (WOG) yielded the highest WK at 283.00 ± 6.21 g, while the treatment with 175 ml of GA3 per hectare (175GA3) produced the lowest weight at 270.83 ± 5.97 g. However, the results indicated that the weight of 1000 kernels was not significantly affected by the different concentrations of GA3. In Table 3, the WK response across different corn varieties ranged from 272.5 g to 281.08 g. NK6 exhibited the highest WK with a mean of 281.08 ± 4.29 g, while NK8 displayed the lowest weight with a mean of 272.50 ± 4.29 g. Similarly, the results demonstrated a non-significant effect of different corn varieties on WK. Table 4 illustrates the interaction effect of WK influenced by different concentrations of GA3 and various corn varieties. Among all the treatments, there were no significant effects on WK. However, the combination of 200 ml of GA3 per hectare with NK6 (200GA3 + NK6) achieved the highest WK with a mean of 287.67 ± 10.88 g, while the combination of 175 ml of GA3 per hectare with NK8 (175GA3 + NK8) resulted in the lowest WK with a mean of 262.00 ± 14.79 g.

Overall, these findings suggest that the applied concentrations of GA3 and the different corn varieties did not significantly impact the weight of 1000 kernels. This indicates that neither the application of GA3 nor the variety of corn significantly affects WK, highlighting the potential stability of WK under the various agricultural conditions studied. Nevertheless, these outcomes emphasise the importance of considering broader environmental effects and farm management practices, which may play vital roles in determining WK results in corn production.

Computed Yield

Table 2 reveals that GA3 has a highly significant positive impact on corn yield. The treatment with 200 ml/ha of GA3 achieved the highest mean yield of 2600.67 ± 460.88 kg, while the control group (WOG) yielded only 1673.67 ± 466.13 kg. This finding is consistent with Singh & Singh (2019), who stated that GA3 enhances crop productivity. Additionally, Table 3 presents significant varietal differences in computed yield (CY). The NK6 hybrid corn recorded the highest yield at 2439.58 ± 299.79 kg, whereas the NK8 variety produced the lowest mean yield of 1840.00 ± 299.79 kg.

Interestingly, the interaction effects illustrated in Figure 2 indicate that the combination of 200 ml/ha GA3 with either the NK6 or NK8 variety resulted in the highest mean yields of 2683.70 ± 543.89 kg and 2619.26 ± 479.45 kg, respectively. This implies that the application of GA3 in conjunction with appropriate varieties significantly boosts yield, supported by research on GA3's role in crop development (Rosales & Galinao, 2018). However, it is important to note that the experiment was notably affected by drought conditions during May and June, which impacted the development of corn ears and their maturity.

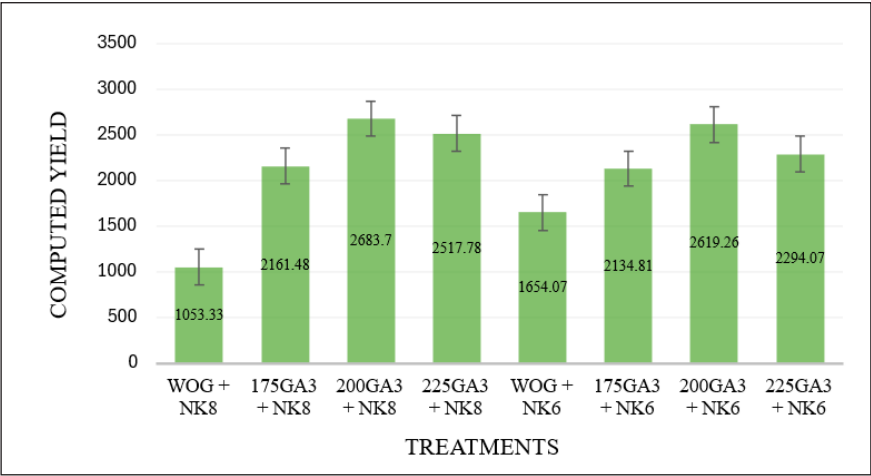


Figure 2. Interaction of effects of hybrid corn varieties applied with different concentrations of GA3 on computed yield

Note. WOG = Without GA3; 175GA3 = 175 ml of GA3 per hectare; 200GA3 = 200 ml of GA3 per hectare; 225GA3 = 225 ml of GA3 per hectare; NK8 = NK8840 Corn Variety; NK6 = NK6640 Corn Variety

This aligns with findings by Zhao et al. (2017) and Khalili et al. (2013), indicating that environmental stressors like drought significantly reduce corn yield, particularly during critical stages such as silking.

CONCLUSION

The study found that GA3 significantly affects both the computed yield and biomass in hybrid corn varieties. A concentration of 200 ml of GA3 per hectare yielded the highest performance, indicating it as the optimal treatment, although other parameters did not show significant differences. The NK6410 variety exhibited superior agronomic characteristics. Notably, the combination of 200 ml/ha GA3 with the NK6 variety produced the highest overall yield, demonstrating a promising synergy for optimising productivity in corn. Therefore, it is advisable to implement this specific treatment strategy to enhance corn production in similar agroecological settings. For more conclusive results, future research should consider multi-season trials to assess the performance of GA3, conducting the same study during the wet season, and conducting multi-location trials with additional corn hybrids to evaluate their responses.

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REFERENCES

- Abrahams, P., Beale, T., Cock, M., Corniani, N., Day, R., Godwin, J., Murphy, S., Richards, G. & Vos, J. (2017). Fall armyworm status impacts and control options in Africa: Preliminary evidence note. *Centre for Agriculture and Bioscience International (CABI), UK*. https://doi.org/10.1564/v28_oct_02
- Anjum, S. A., Ashraf, U., Zohaib, A., Tanveer, A., Naeem, M., Ali, I., & Nazir, U. (2017). Growth and development responses of crop plants under drought stress: A review. *Zemdirbyste-Agriculture*, 104(3), 267-276. <https://doi.org/10.13080/z-a.2017.104.034>
- Ayyub, C. M., Manan, A., Pervez, M. A., Ashraf, M., Afzal, M., Ahmed, S., & Shaheen, M. R. (2013). Foliar feeding with gibberellic acid (GA₃): A strategy for enhanced growth and yield of okra (*Abelmoschus esculentus* L. Moench.). *African Journal of Agricultural Research*, 8(25), 3299-3302.
- Camara, M. C., Vandenbergh, L. P. S., Rodrigues, C., de Oliveira, J., Faulds, C. B., Bertrand, E., & Soccol, C. R. (2018). Current advances in gibberellic acid (GA₃) production, patented technologies and potential applications. *Planta*, 248(5), 1049-1062. <https://doi.org/10.1007/s00425-018-2959-x>
- El-Nwehy, S. S., & Afify, R. R. M. (2023). Utilisation of gibberellic acid (GA₃) and mepiquat chloride (M.C) as growth regulators on maize to alleviate salinity stress. *SABRAO Journal of Breeding and Genetics*, 55(5), 1654-1665. <https://doi.org/10.54910/sabrao2023.55.5.18>
- Ge, N., Jia, J. S., Yang, L., Huang, R. M., Wang, Q. Y., Chen, C., & Chen, J. W. (2023). Exogenous gibberellic acid shortening after-ripening process and promoting seed germination in a medicinal plant *Panax notoginseng*. *BMC Plant Biology*, 23(1), Article 67. <https://doi.org/10.1186/s12870-023-04084-3>
- Giller, K. E., Delaune, T., Silva, J. V., Descheemaeker, K., Van De Ven, G., Schut, A. G., & van Ittersum, M. K. (2021). The future of farming: Who will produce our food? *Food Security*, 13(5), 1073-1099. <https://doi.org/10.1007/s12571-021-01184-6>
- Khalili, M., Naghavi, M. R., Aboughadareh, A. P., & Rad, H. N. (2013). Effects of drought stress on yield and yield components in maize cultivars (*Zea mays* L.). *International Journal of Agronomy and Plant Production*, 4(4), 809-812. <https://www.cabidigitallibrary.org/doi/full/10.5555/20133138197>
- Mukarram, M., Mohammad, F., Naeem, M., & Khan, M. M. A. (2021). Exogenous gibberellic acid supplementation renders growth and yield protection against salinity induced oxidative damage through upregulating antioxidant metabolism in fenugreek (*Trigonella foenum-graecum* L.). In M. Naeem, M. M. A. Khan, & M. Mukarram (Eds.), *Fenugreek: Biology and applications* (pp. 99-117). Springer. https://doi.org/10.1007/978-981-16-1197-1_6
- Noda-Leyva, Y., & Martín-Martín, G. J. (2017). Efecto de la distancia de siembra en el rendimiento de *Morus alba* (L.) var. yu-12 [Effect of planting distance on the yield of *Morus alba* (L.) var. yu-12]. *Pastos y Forrajes*, 40(1), 23-28. <https://www.redalyc.org/articulo.oa?id=269150990003>

- Ocampo, A. M., Labios, R. V., Labios, J. D., Lapoot, C. R., Tumamang, S. C., Gines, H. C., & Witt, C. (2015). Site-specific nutrient management for maize production in favorable environments of the Philippines. *Philippine Journal of Crop Science*, 40(3), 40-48. <https://www.cabidigitallibrary.org/doi/full/10.5555/20163025045>
- Philippine Competition Commission. (2021). *Issues paper on corn industry in the Philippines*. <https://www.phcc.gov.ph>
- Philippine Statistics Authority. (2024). *Updates on April-June 2024 palay and corn estimates based on standing crop*. <https://www.psa.gov.ph>
- Ramey, E. A. (2010). Seeds of change: Hybrid corn, monopoly, and the hunt for superprofits. *Review of Radical Political Economics*, 42(3), 381-386. <https://doi.org/10.1177/0486613410378005>
- Rauf, F., Ullah, M., Kabir, M., Mia, M., & Rhaman, M. (2022). Gibberellic acid enhances the germination and growth of maize under salinity stress. *Asian Plant Research Journal*, 10(3), 5-15. <https://doi.org/10.9734/aprj/2022/v10i3191>
- Reyes, C., Domingo, M. S. N., Mina, C. D., & Gonzales, K. G. (2009). *Climate variability, SCF, and corn farming in Isabel, Philippines: A farm and household level analysis* (Discussion Paper Series No. 2009-04). Philippine Institute for Development Studies.
- Rosales, R. J. G., & Galinato, R. G. (2018). Effect of gibberellic acid on the flowering and yield performance of hybrid variety of *Momordica charantia*. *International Journal of Research in Agricultural Sciences*, 5(6), 105-109. https://ijras.org/administrator/components/com_jresearch/files/publications/IJRAS_710_FINAL1.pdf
- Rustia, A. S., Guiriba, D. N. S., Bautista, K. A. D., Mahoney, D., Barrios, E. B., Villarino, C. B. J., & Capanzana, M. V. (2022). Risk profiling of fumonisin in corn (*Zea mays* L.) to the Filipino consuming population. *Philippine Journal of Science*, 151(1), 73-93. <https://doi.org/10.56899/151.01.06>
- Shiferaw, B., Prasanna, B. M., Hellin, J., & Bänziger, M. (2011). Crops that feed the world 6. Past successes and future challenges to the role played by maize in global food security. *Food Security*, 3(3), 307-327. <https://doi.org/10.1007/s12571-011-0140-5>
- Singh, S., & Singh, T. (2019). Effect of gibberellic acid on growth, yield and quality parameters of chilli (*Capsicum annuum* L.). *Journal of Pharmacognosy and Phytochemistry*, 8(2), 2021-2023. <https://www.phytojournal.com/archives/2019/vol8issue2/PartAH/8-2-87-356.pdf>
- Solaimalai, A., Anantharaju, P., Irulandi, S., & Theradimani, M. (2020). *Maize crop: Improvement, production, protection and post-harvest technology*. CRC Press. <https://doi.org/10.1201/9781003090182>
- Zaman, M., Kurepin, L. V., Catto, W., & Pharis, R. P. (2015). Enhancing crop yield with the use of N-based fertilisers co-applied with plant hormones or growth regulators. *Journal of the Science of Food and Agriculture*, 95(9), 1777-1785. <https://doi.org/10.1002/jsfa.6938>
- Zhao, C., Liu, B., Piao, S., Wang, X., Lobell, D. B., Huang, Y., & Asseng, S. (2017). Temperature increase reduces global yields of major crops in four independent estimates. *Proceedings of the National Academy of Sciences*, 114(35), 9326-9331. <https://doi.org/10.1073/pnas.1701762114>

Review Article

Systematic Scoping Literature Review on Black Soldier Fly Larvae Frass Application Methods and Rate

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ABSTRACT

The growing demand for sustainable agriculture and effective organic waste management has increased interest in the use of black soldier fly larvae (BSFL) frass. It has a high content of nitrogen, phosphorus, potassium, organic carbon, and beneficial microbial communities. However, the frass application methods and rate are not clearly stated. Thus, this review objective is to determine the frass application methods and rate. This review synthesises data from 16 studies conducted across various soil types, climates, and cropping systems to evaluate the agronomic, ecological, and microbiological effects of BSFL frass application. Direct soil incorporation is most common and effective for the soil and plant benefit. Frass improves plant and soil health. Low-to-moderate frass application rates (2-25 t/ha) enhance plant growth, nutrient uptake, and yield, particularly in nutrient-poor soils. However, higher application rates can cause phytotoxic effects, salinity stress, and microbial imbalance. This emphasises the importance of identifying crop- and context-specific optimum doses. Despite promising outcomes, significant research gaps remain, such as long-term field validation, interactions with diverse soil microbiomes, the fate of potentially harmful elements like heavy metals, and standardised processing and quality control of frass.

Keywords: Black soldier fly larvae frass, BSFL frass; circular bioeconomy systems; insect-based frass; soil amendment

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INTRODUCTION

The global agricultural sector is gradually challenged by the need to produce more food while reducing dependence on synthetic fertilisers. Synthetic fertiliser is not only

energy-intensive to produce but also contributes to environmental degradation, including soil acidification, nutrient runoff, and greenhouse gas emissions (Sabina et al., 2025). Simultaneously, the accumulation of organic waste from urban, agricultural, and agro-industrial sources presents a major sustainability concern. Insect-based bioconversion, particularly using the Black Soldier Fly (BSFL; *Hermetia illucens*) has emerged recently as a promising circular bioeconomy solution that addresses both waste management and sustainable fertiliser production.

Black soldier fly larvae (BSFL) can efficiently convert a wide range of organic residues into high-value insect biomass (for use in animal feed), insect oil and a nutrient-rich frass (Abd El-Hack et al., 2020; Gurung et al., 2024; Huang et al., 2024; Oteri et al., 2021; Putri et al., 2023; Quigley et al., 2025; Watson et al., 2021). BSFL frass is comprised of undigested substrate, insect excreta, shed exoskeletons, and associated microorganisms (Abd Manan et al., 2024). With this, it has gained attention as a potential biofertiliser due to its essential plant nutrient content (N, P, K), organic matter, and beneficial microbes (Abd Manan et al., 2024). Early studies suggest that frass application can enhance plant growth, improve soil fertility, and stimulate microbial activity (Beesigamukama, Mochoge, Korir, Fiaboe, et al., 2020; Beesigamukama, Mochoge, Korir, Musyoka, et al., 2020). Frass is a viable alternative to synthetic fertilisers and compost in both organic and conventional farming systems.

Despite growing interest, the agronomic performance of BSFL frass varies widely depending on factors such as larval diet, frass processing method, application rate, soil type, and crop species (Lomonaco et al., 2024; Malheiro et al., 2024; Watson et al., 2021). Research to date has been fragmented. It is often limited to short-term or greenhouse trials and lacks standardised methodologies. There is a need to synthesise existing findings to understand the conditions under which BSFL frass is most effective, identify potential limitations or risks (e.g., salinity, heavy metal accumulation), and determine optimal application strategies.

This paper compiles and analyses recent experimental studies on BSFL frass use in agriculture, comparing its effects on plant productivity, soil chemical and biological properties, and environmental outcomes. By identifying patterns, benefits, and trade-offs across diverse systems, the review aims to find out the black soldier fly larvae frass application to soil and clarify the current knowledge landscape. Also, this paper highlights research gaps and proposes directions for the sustainable and scalable use of insect frass in soil fertility management.

This review addresses the following three research questions:

- What methods of BSFL frass application to soil and plants are used in current experimental studies?
- What application rates of BSFL frass have been reported for soil and plant systems?

- What effects do different application rates and methods have on plant productivity and soil properties?

THEORETICAL FRAMEWORK

Soil Fertility and Nutrient Use Efficiency (NUE)

Soil fertility is the soil's capacity to supply essential nutrients to plants in adequate amounts and proper balance (Havlin, 2020). BSFL frass contains macronutrients (N, P, K) and micronutrients (e.g., Fe, Zn, Mn) critical for plant development. The Nutrient Use Efficiency (NUE) theory supports that the form, timing, and availability of nutrients determine plant uptake efficiency and crop productivity (Sarkar & Baishya, 2017).

BSFL frass provides readily available nitrogen (as NH_4^+ and NO_3^-), organic carbon that improves soil structure and microbial activity, phosphorus, and potassium in slow-release forms (Beesigamukama, Mochoge, Korir, Fiaboe, et al., 2020; Chavez et al., 2023; Malheiro et al., 2024). When BSFL frass is applied at optimal rates, it can match or exceed the performance of synthetic fertilisers in enhancing plant nutrient uptake and yield, while minimising nutrient losses to the environment.

Organic Matter Dynamics and Soil Health

BSFL frass is rich in organic matter, which contributes to the formation of soil aggregates, water-holding capacity, and cation exchange capacity (Bashir et al., 2021; Menino et al., 2021). The soil health model recognises that organic inputs can restore degraded soils by supporting biological activity, reducing erosion, and improving nutrient cycling (Culas et al., 2025). The incorporation of frass into soil stimulates microbial processes that decompose organic residues, release nutrients, and enhance microbial biomass and enzymatic activity (Salomon et al., 2025). Repeated frass application can improve long-term soil health by building soil organic carbon and supporting a more resilient microbial ecosystem (Ashworth et al., 2025; Salomon et al., 2025).

Microbial Ecology and Soil-Plant-Microbe Interactions

BSFL frass introduces microbial populations and stimulates existing communities in the rhizosphere (Gurung et al., 2024). According to microbial ecology theory, changes in microbial diversity and function can influence nutrient availability, plant growth promotion, and pathogen suppression (Ambrosini et al., 2016; Chen et al., 2024; Meena et al., 2017). For instance, beneficial genera (e.g., *Bacillus*, *Actinobacteria*) may be enhanced, and functional gene expression (e.g., *nirK*, *nosZ*) may be stimulated, affecting nitrogen cycling (Lomonaco et al., 2024; Rummel et al., 2021). The biological activity within frass mediates

positive feedback loops between soil microbes and plants, leading to improved nutrient uptake and plant stress tolerance (Poveda, 2021).

Waste Valorisation and Circular Economy Theory

The use of BSFL frass is aligned with circular economy principles, which emphasise the transformation of waste into valuable resources to reduce environmental impact and close nutrient loops (Abd Manan et al., 2024). Organic wastes (e.g., food scraps, manure, brewery waste) are converted by BSFL into insect biomass and frass (Carroll et al., 2023; Gurung et al., 2024; Wu et al., 2023). Frass returns nutrients to the soil, reducing reliance on mined or industrially produced fertilisers (Amorim et al., 2024). BSFL frass serves as a functional bridge between waste management and sustainable agriculture, enabling resource efficiency and reducing external inputs in farming systems (Abd Manan et al., 2024).

Dose-Response and Environmental Thresholds

The dose-response model in environmental science explains that while low-to-moderate rates of input may be beneficial, higher concentrations can lead to toxicity, salinity stress, or ecological disruption (Chele et al., 2021). Over-frass application may increase soil electrical conductivity (EC), alter pH, or introduce heavy metals (e.g., Zn, Cu) from substrate residues (Watson et al., 2021).

Microbial diversity may decline at high application rates, potentially accompanied by shifts toward fast-growing (r-strategists) microbial taxa (Maron et al., 2018). There exists an optimum frass application threshold beyond which benefits plateau or reverse, emphasising the need for careful dose calibration.

METHODOLOGY

Literature Survey Methodology

Based on the PRISMA guideline, a scoping literature review, thematic analysis, and tabular analysis were adopted (Moher et al., 2009). The review intended to address the three research questions: What application rates of black soldier fly larvae (BSFL) frass have been reported for soil and plant systems? What methods of BSFL frass application to soil and plants are used in current experimental studies? What effects do different application rates and methods have on plant productivity and soil properties? Research Registry and OSF (Open Science Framework) were searched to ensure a similar scoping review study protocol was not registered. There was no previous research found that addressed the present area of interest.

Search Strategy

The electronic scoping search will be conducted in mid-May 2025. The databases searched included PubMed, ScienceDirect, Scopus, and Emerald Insight. The current review covers four major research subjects, including livestock, aquaculture, and agronomy studies. Besides, two main variables using Medical Subject Headings (MeSH) and keywords are applied in this study, as shown in Table 1 and Supplementary Materials (the detailed keywords search, especially for ScienceDirect, since the maximum number of keywords searched is 8). The search string is applied only to the title, abstract, and keywords.

Table 1
Keywords used in the database search

Black Soldier Fly		Black Soldier Fly Larvae Frass		Application Rate
		"black soldier fly larvae frass"		"application rate"
		OR		OR
"Hermetia illucens"		"bsfl frass"		"soil to frass ratio"
OR		OR		OR
"black soldier fly"	AND	"black soldier fly frass"	AND	"frass to soil ratio"
OR		OR		OR
bsf		"bsf frass"		"frass percentage"
		OR		OR
		"insect frass"		"frass-soil mix*"

Note. The asterisk (*) indicates truncation in the search strategy, allowing retrieval of records containing different endings of the root word. For example, mix* includes mix, mixed, mixing, and mixture

Inclusion Criteria

The inclusion and exclusion criteria of the scoping review included (a) language of the studies: studies published in the English language are included. Studies published in a language other than English are excluded. (b) Type of studies: Formal research studies, including journal articles, were only reviewed to ensure the quality of the included studies. Review articles, including narrative and systematic reviews, were excluded to ensure that only primary, empirical studies reporting original research findings were synthesised. This approach was taken to avoid duplication of data and maintain a consistent methodological basis for comparison across included studies. (c) The focus of the studies: based on the study’s objective, the included studies must focus on the black soldier fly frass application rate. Studies focusing on anything other than black soldier fly feeding and growth are excluded. (d) Quality assessment: the selected studies should pass the quality assessment of the BEME framework as described in the quality assessment section to ensure the quality of the scoping review. The excluded studies are either irrelevant or failed the quality assessment of the BEME. Figure 1 shows the search flowchart.

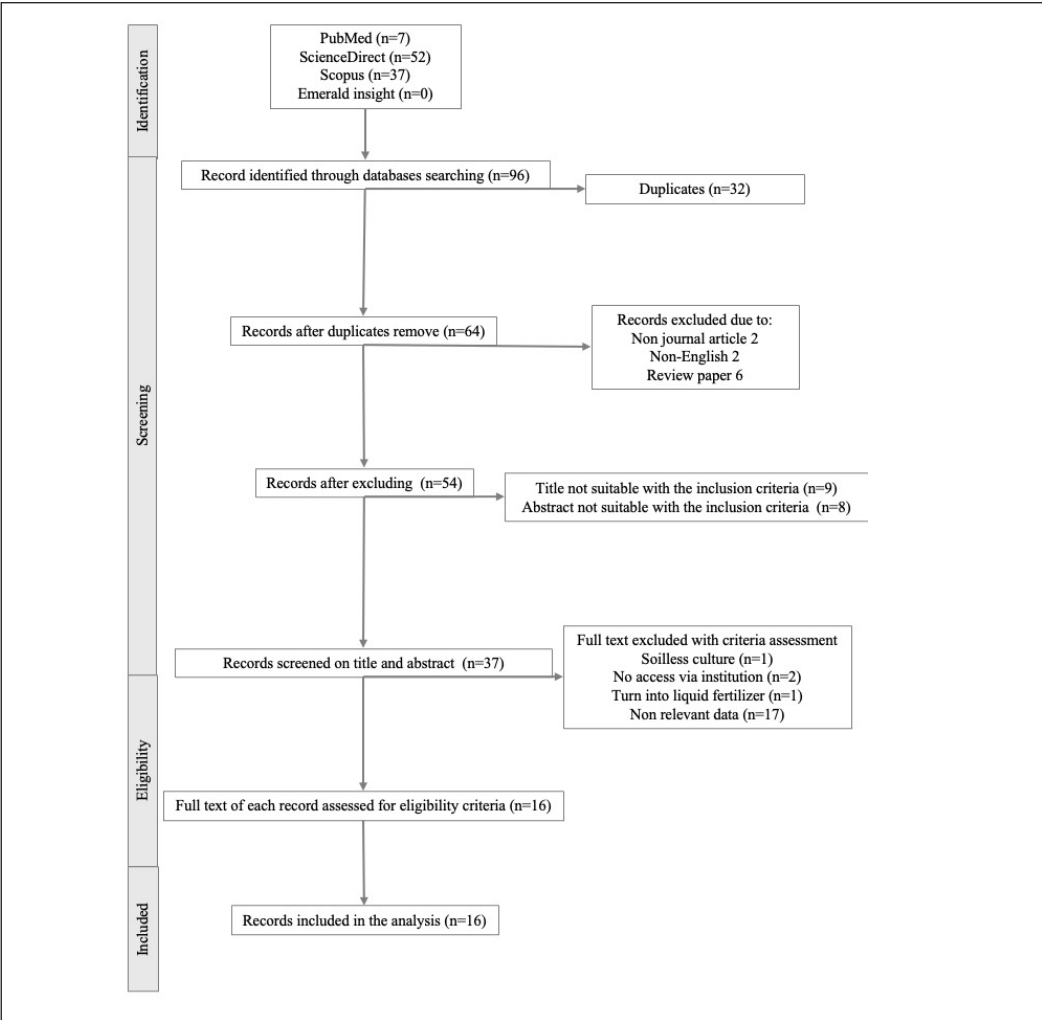


Figure 1. PRISMA flow diagram

Study Quality Assessment

The quality assessment tool is critical in detecting the quality of the not rigorously conducted. The BEME quality framework is used to evaluate the quality of the selected studies (Buckley et al., 2009). The BEME quality framework included 11 indicators for quantitative studies. It is provided with a consistent approach to assessing the selected studies. Each indicator in the BEME is evaluated as 1 = met or 0 = unmet. Each study should complete a minimum of seven indicators to be deemed high quality. Studies scoring below three are rated as weak, studies scoring four to seven are rated as moderate, and those scoring seven and above are rated as strong and included (Buckley et al., 2009). The process of quality assessment is shown in Table 2 and Supplementary Materials.

Table 2
Average BEME quality assessment results from two authors, who followed the guidelines (Buckley et al., 2009)

Quality Indicator	Details	Beesigamukama et al. (2020)	Beesigamukama et al. (2020)	Borkent and Hodge (2021)	Carroll et al (2023)	Chavez et al. (2023)	Chiam et al. (2021)	Gurung et al. (2024)	Huang et al. (2024)	Malheiro et al. (2024)	Mostafaie et al. (2025)	Putri et al. (2023)	Rodgers et al. (2024)	Rummel et al. (2021)	Sawinska et al. (2024)	Watson et al. (2021)	Wu et al. (2023)
Research question	Is the research question (s) or hypothesis clearly stated?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Study subjects	Is the subject group appropriate for the study being carried out (number, characteristics, selection, and homogeneity)?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
'Data' collection methods	Are the methods used (qualitative or quantitative) reliable and valid for the research question and context?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Completeness of 'data'	Have subjects dropped out? Is the attrition rate less than 50%? For questionnaire-based studies, is the response rate acceptable (60% or above)?	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

Table 2 (continued)

Quality Indicator	Details	Beesigamukama et al. (2020)	Beesigamukama et al. (2020)	Borkent and Hodge (2021)	Carroll et al (2023)	Chavez et al. (2023)	Chiam et al. (2021)	Gurung et al. (2024)	Huang et al. (2024)	Malheiro et al. (2024)	Mostafaie et al. (2025)	Putri et al. (2023)	Rodgers et al. (2024)	Rummel et al. (2021)	Sawinska et al. (2024)	Watson et al. (2021)	Wu et al. (2023)
Control for confounding	Have multiple factors/ variables been removed or accounted for where possible?	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
Analysis of results	Are the statistical or other methods of results analysis used appropriately?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Conclusions	Is it clear that the data justify the conclusions drawn?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Reproducibility	Could the study be repeated by other researchers?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Prospective	Does the study look forward in time (prospective) rather than backward (retrospective)?	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1
Ethical issues	Were all relevant ethical issues addressed?	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
Triangulation	Were the results supported by data from more than one source?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Total		9	9	9	8	8	8	10	10	10	10	10	10	10	10	10	10

Search Outcome

A total of 96 studies were identified from the four electronic databases, as shown in the Supplementary Materials. After extracting duplicated studies and including studies published in English, a total of 54 studies remained, of which 37 were excluded based on the title and abstract screening. A text of 16 studies was screened, from which 4 studies were excluded as they included no relevant data (n=17).

RESULTS AND DISCUSSIONS

Description of the Selected Studies

The selected studies were 16 based on the selected keywords. The utilisation of Black Soldier Fly (BSF) frass as a soil amendment and organic fertiliser across diverse geographical, agronomic, and soil conditions was studied in the past 5 years from 2020 (Table 3). Since no time frame is specified, the scientific studies of frass application on soil available are limited. The studies ranged from controlled pot and greenhouse experiments to field trials. The selected study involved a wide array of crops, including cereals (maize, oats, barley), vegetables (lettuce, tomato, broccoli, chilli), and herbs (basil, parsley). To produce frass, the selected studies used brewery waste, food and kitchen residues, oil palm waste, and mixed manures as larvae fed and reflected a wide spectrum of nutrient profiles and potential environmental implications.

Frass application rates ranged broadly, from nutrient-equivalent applications (e.g., 0.03-0.1 t/ha on an N basis) to volume- or weight-based doses as high as 187 t/ha in some pot trials. The impacts of frass application were measured on plant growth parameters (biomass, nutrient uptake, yield), soil nutrient dynamics (e.g., N mineralisation, EC, pH, organic matter), and soil biological responses (e.g., microbial diversity, functional gene expression, presence of beneficial or pathogenic taxa).

Effect of Frass Application Methods

BSFL frass has been applied using diverse experimental approaches (Table 4). They ranged from direct soil incorporation and N-equivalent dosing to combined mineral fertiliser strategies and soil incubation studies. Direct basal application remains the most agronomically relevant method, while nutrient-equivalent and combined approaches facilitate comparison with conventional fertilisers. Pot and incubation studies provide mechanistic insights but require careful extrapolation to field conditions.

Different BSFL frass application methods exert distinct effects on plant productivity and soil properties (Table 5). Direct soil incorporation at moderate rates most consistently enhanced crop yield and soil fertility, particularly in degraded soils.

Table 3
Selected studies related to Black Soldier Fly Larvae frass and the optimum application rate

Author (Year)	Location	Soil Type	Larval Diet	Crop(s)	Application Rate (t/ha)	Plant Effects	Soil Effects	Soil Organism Effects	Optimum Rate (t/ha)
(Beesigamukama, Mochoge, Korir, Musyoka, et al., 2020)	Nairobi, Kenya	Acric Ferralsols (acidic, low OM)	Brewery waste	Maize	0.03, 0.06, 0.1 (N basis)	Yield 20-27% higher than SAFI; best at 100 kg N/ha	Fast N release, late-season deficiency	Not reported	0.03 t N ha ⁻¹ recommended; 0.1 t N ha ⁻¹ highest yield
(Beesigamukama, Mochoge, Korir, Fiaboe, et al., 2020)	Nairobi, Kenya	Acric Ferralsols (acidic, shallow, low OM)	Brewery waste	Maize	0, 1.4, 2.9, 4.8	Best yield at 7.5 t/ha; better NUE than SAFI/urea	Not reported	Not reported	2.5 t/ha + 30 kg N/ha
(Borkent & Hodge, 2021)	Dublin, Ireland	High-nutrient compost & low-nutrient mix	Brewery waste	Basil, lettuce, etc.	0.1-0.8	Growth improved in 8/10 crops; basil and lettuce were best	Stronger effect in high-nutrient soils	Not reported	0.4-0.7
(Carroll et al., 2023)	Dublin, Ireland	Low-/high-nutrient potting mix	Brewery waste	Oats, barley, spelt	0.2-1.6	Growth improved; best at 6-8 g/pot; >8g reduced survival	Effect greater in low-nutrient soil	Not reported	0.6-0.8
(Chavez et al., 2023)	Colorado, USA	Peat mix	Distillery grains	Tomato	22.5, 45, 90	No significant yield gain; 40% reduced Brix and biomass	High NPK, pH 9.5, micronutrient toxicity risk	Not reported	22.5-45

Table 3 (continued)

Author (Year)	Location	Soil Type	Larval Diet	Crop(s)	Application Rate (t/ha)	Plant Effects	Soil Effects	Soil Organism Effects	Optimum Rate (t/ha)
(Chiam et al., 2021)	Singapore	Sandy loam	Chicken feed → okara	Lettuce	10, 20, 30	10% frass + fert = max biomass; 30% reduced growth	High N, C; reduced soil pH	30% reduced diversity; 10% preserved microbial health	10
(Gurung et al., 2024)	Western Australia	Coarse sand	Mixed animal wastes	Chilli	1.7-10	Best growth at 0.6%; reduced at higher rates	NH ₄ ⁺ /NO ₃ ⁻ increased; high rates raised pH/EC	0.6% boosted beneficial microbes; 1.2% reduced benefits	5.0
(Huang et al., 2024)	Guangdong, China	Haplic Acrisol	Kitchen waste	Not specified	0-70	Not specified	EC, NH ₄ ⁺ , NO ₃ ⁻ , DOC ↑; nutrients declined over 28 days	Bacterial diversity ↑ at ≥2.5%; fungi ↓ at high rates	35
(Malheiro et al., 2024)	Portugal	Sandy loam (Lufa 2.2)	Vegetable by-products + cereals	Onion, Turnip, Tomato	1.6-99.8	Low-mid rates helped; high rates inhibited germination	OM, K, P, pH ↑; salinity is low overall	Enchytraeids ↑, Collembolans are unaffected	Turnip: 12.5-25; Onion: 12.5; Tomato ≤12.5
(Mostafaie et al., 2025)	Portugal	Loamy sand (Lufa 2.2)	Olive pomace + Gainesville feed	Ryegrass, Broccoli	5.85-187.2	2.4-4.8% improved root traits; 9.6% phytotoxic	Not reported	Not reported	46.8-93.6

Table 3 (continued)

Author (Year)	Location	Soil Type	Larval Diet	Crop(s)	Application Rate (t/ha)	Plant Effects	Soil Effects	Soil Organism Effects	Optimum Rate (t/ha)
(Putri et al., 2023)	Indonesia	Not specified	Oil palm waste	Red amaranth	120-600	Best at 10% (120 t/ha); 30-50% reduced growth	10% improved fertility; 30-50% caused metal toxicity	Not reported	120
(Rodgers et al., 2024)	Dublin, Ireland	Low-nutrient mix	Brewery waste	Ryegrass, Timothy	0.125-0.75 (N basis)	Timothy & PRG grew better; Timothy is best at a high rate	Not reported	Not reported	>0.75 (needs further testing)
(Rummel et al., 2021)	Germany	Haplic Luvisol	Carb/ protein-based frass	Not specified	0.127-0.422 (N basis)	Not specified	Protein-frass: N mineralisation; Carb-frass: N immobilisation	GHG emissions ↑; microbial biomass ↑	~14 (low emission), ~43 (high performance)
(Sawinska et al., 2024)	Poland	Commercial OM-rich soil	BSF frass	Lettuce	1.6-2.0	Max yield 30.4 g/pot at 12.5 g frass	Not reported	Not reported	2
(Watson et al., 2021)	Germany	Sandy loam	Wheat bran	Not applicable	48.75-97.5	Not reported	High OM; nutrient-rich; heavy metal toxicity mitigated	Stimulated fungi & microbes	97.5
(Wu et al., 2023)	Not specified	Not specified	Food waste	Maize	21-126	Biomass ↑ at 1-4%; ↓ at 6% (salinity)	Not reported	Not reported	42

Note. *SAFI: commercial organic fertiliser made from composted chicken manure, biochar, and rock phosphate in Mwea town, Kirinyaga County, Kenya

Table 4
Methods of BSFL frass application to soil and plants in experimental studies

Application Method	Description	Unit/ Expression of Rate	Typical Experimental setup	Representative Studies	Main Purpose/Rationale
Direct soil incorporation (basal application)	Frass is mixed uniformly into the soil or growth medium before planting	t/ha (field); % w/w or g/pot (pots)	Field plots, pots, greenhouse trials	(Beesigamukama, Mochoge, Korir, Fiaboe, et al., 2020; Beesigamukama, Mochoge, Korir, Musyoka, et al., 2020; Chiam et al., 2021; Gurung et al., 2024; Malheiro et al., 2024; Wu et al., 2023)	Mimics farmer practice; evaluates effects on crop growth, soil fertility, salinity, and microbial communities
Nutrient-equivalent (N-based) application	Frass applied according to its N content, aligned with recommended fertiliser N rates	kg N/ha or frass mass equivalent	Field and pot trials with fertiliser comparisons	(Beesigamukama, Mochoge, Korir, Musyoka, et al., 2020; Rodgers et al., 2024; Rummel et al., 2021)	Enables direct comparison with mineral fertilisers; assesses nutrient use efficiency (NUE)
Combined frass + mineral fertiliser	Frass applied at moderate rates with supplemental mineral N or commercial fertiliser	t/ha frass + kg N/ha; %	Field and pot experiments	(Beesigamukama, Mochoge, Korir, Fiaboe, et al., 2020; Chiam et al., 2021)	Integrated nutrient management maximises yield while reducing fertiliser inputs and toxicity risks
Percentage or mass-based pot application	Frass added as a proportion of soil mass/volume or as g per pot	% w/w; g/pot (converted to t/ha)	Controlled pot and greenhouse studies	(Borkent & Hodge, 2021; Carroll et al., 2023; Mostafaei et al., 2025; Sawinska et al., 2024)	Allows precise dosing and mechanistic assessment; useful for threshold and toxicity studies
Soil incubation (no plants)	Frass mixed into the soil and incubated without crops	% w/w or kg N/ha	Laboratory incubation studies	(Huang et al., 2024; Rummel et al., 2021)	Isolates soil biochemical and microbial responses; assesses N mineralisation and GHG emissions

Table 5
Effects of BSFL frass application methods on plant productivity and soil properties

Application Method	Effects on Plant Productivity	Effects on Soil Chemical Properties	Key Advantages	Key Limitations/Risks
Direct soil incorporation (basal application)	Consistent increases in biomass and yield at moderate rates (10-45 t/ha); Improved nutrient uptake and NUE; Yield suppression at excessive rates	↑ Mineral N (NH ₄ ⁺ , NO ₃ ⁻), P, K, organic C; Improves fertility in acidic, nutrient-poor soils; High rates ↑ EC and risk salinity	Practical and compatible with conventional fertiliser management; supports both plant growth and soil fertility	Over-application causes phytotoxicity, salinity stress, and nutrient imbalance
Nutrient-equivalent (N-based) application	Yield comparable to mineral fertilisers (e.g., urea, SAFI) High NUE and early vigour; Possible late-season N limitation	Rapid N mineralisation; May improve in soil organic matter	Allows direct fertiliser comparison; efficient nutrient delivery	Does not fully capture the soil-conditioning benefits of frass
Combined frass + mineral fertiliser	Highest and most stable yields; Reduced yield variability compared with frass alone	Balanced nutrient supply; Lower EC and reduced risk of toxicity	Optimises productivity and soil health; supports integrated nutrient management	Requires careful rate optimisation and fertiliser access
Percentage / mass-based pot application	Strong growth response at low-moderate percentages (≤5%); Growth inhibition at high percentages (>5-10%)	Rapid nutrient release; High rates sharply increase EC and pH	High experimental control; identifies toxicity thresholds	Field-equivalent rates often unrealistically high; limited extrapolation
Soil incubation (no plants)	Not assessed	Clarifies N mineralisation vs. immobilisation; High rates increase CO ₂ and N ₂ O emissions	Reveals mechanisms driving soil responses	Cannot directly predict crop yield responses

Nutrient-equivalent applications often produced yields comparable to mineral fertilisers while also contributing additional soil-conditioning benefits, although nutrient release tends to be slower than synthetic fertiliser. Combined frass-mineral fertiliser strategies often provided a more balanced nutrient supply and supported crop productivity while potentially reducing risks associated with salinity and nutrient imbalance risks when applied at appropriate rates. Pot-based and incubation approaches clarified response thresholds and biogeochemical mechanisms but require cautious extrapolation to field conditions.

Effect of Frass Application Rate

Based on most of the selected studies, moderate application rates of BSFL frass improved plant growth and nutrient use efficiency (NUE), often bettering or equaling conventional fertilisers like urea or SAFI (semi-arid fertiliser input) (Beesigamukama, Mochoge, Korir, Fiaboe, et al., 2020; Beesigamukama, Mochoge, Korir, Musyoka, et al., 2020). Generally, frass rates ranging from 10 to 45 t/ha were observed as optimal plant responses, such as enhanced root and shoot biomass, chlorophyll content, and nutrient uptake (Gurung et al., 2024; Putri et al., 2023; Wu et al., 2023). Yet, in controlled pot experiments, the optimal concentration was often expressed as a percentage of soil mass or volume (e.g., 2.4-4.8%), and the amount was generally high when converted to the unit of ton/ha (Putri et al., 2023; Wu et al., 2023).

Frass also had evident positive effects on soil quality, mainly by increasing levels of mineral nitrogen (NH_4^+ , NO_3^-), organic carbon, and essential nutrients (P, K, Zn). In acidic and nutrient-poor soils (e.g., Acric Ferralsols in Kenya or Tenosols in Australia), frass application promptly improved fertility and supported early crop development. Yet, excessive frass application (30t/ha (Chiam et al., 2021); 99.8t/ha (Mostafaie et al., 2025); 360-600t/ha (Putri et al., 2023)) led to negative consequences such as germination *suppression*, reduced biomass, accumulation of heavy metals (e.g., Zn, Mn, Cu, B), and raised soil electrical conductivity (EC), which is often due to salinity stress or nutrient imbalances.

Microbial responses were equally critical. In optimal ranges, frass enhanced microbial biomass carbon (MBC) and nitrogen (MBN), supported beneficial bacterial populations (e.g., Kribbella, Nocardoides), and promoted functional genes related to C and N cycling (Gurung et al., 2024; Huang et al., 2024; Rummel et al., 2021). However, high application rates (30t/ha) disrupted microbial communities by reducing diversity and promoting opportunistic taxa like *Acinetobacter*, which may be linked to reduced plant performance (Chiam et al., 2021; Rummel et al., 2021). In a few studies (Huang et al., 2024; Rummel et al., 2021), frass behaved more like fresh manure than compost, with high CO_2 and N_2O emissions, especially from carbohydrate-rich substrates, indicating a trade-off between fertility gains and environmental sustainability (Huang et al., 2024; Rummel et al., 2021).

Overall, BSFL frass shows robust potential as a sustainable, circular biofertiliser and is suitable for integrated nutrient management in low-input or degraded schemes. When the frass is wisely applied, it can improve crop productivity, soil fertility, and microbial function. Yet to avoid phytotoxicity or ecological disruption, application rates always depended on crop type, soil conditions, and frass composition. Across the selected studies, the optimum frass rate generally fell between 10 and 45 t/ha (Table 6). In contrast, particularly in nutrient-demanding crops, low application rates produced only marginal benefits. Excessive frass inputs (≥ 30 t/ha) resulted in phytotoxic effects, elevated soil electrical conductivity,

and microbial community disruption, indicating that over-application may compromise both productivity and environmental sustainability. Yet, some crop-specific limits diverge significantly depending on soil texture, baseline fertility, and frass quality. The evidence supports BSFL frass as a valuable component in sustainable agriculture and organic waste recycling. Yet future studies should further refine dosing guidelines, assess long-term soil health impacts, and explore synergies with other organic amendments.

Table 6
Effects of BSFL Frass application rates on crops, soil fertility, and soil microorganisms

Application Rate Category	Typical Application Range*	Effects on Crop Growth and Yield	Effects on Soil Fertility	Effects on Soil Microorganisms
Low rate	<10 t/ha (field) ≤2% soil mass/ volume (pot)	Minor or inconsistent improvements in seedling vigour and early growth; Generally inferior to moderate frass rates and mineral fertilisers (e.g., urea, SAFI); Often insufficient to meet crop N demand	Slight increases in available N, P, and K; Minimal changes in soil organic carbon and CEC; Benefits are more apparent in nutrient-poor or acidic soils	Neutral to slightly positive effects on microbial biomass (MBC, MBN); Limited changes in microbial community composition or functional diversity
Moderate rate (optimal)	10-45 t/ha (field-equivalent) ~2.4-4.8% soil mass/ volume (pot)	Significant increases in shoot and root biomass; Higher chlorophyll content and photosynthetic activity; Improved nutrient uptake and nutrient use efficiency (NUE); Often equal to or better than conventional fertilisers	Increased mineral N (NH ₄ ⁺ , NO ₃ ⁻) and soil organic carbon; Enhanced availability of P, K, and micronutrients (e.g., Zn); Improved fertility in degraded or low-input soils without excessive EC	Increased microbial biomass carbon and nitrogen; Enrichment of beneficial taxa (e.g., Kribbella, Nocardioides); Enhanced functional genes related to C and N cycling
High rate	≥30 t/ha (field) >5% soil mass/ volume (pot) Extreme cases: 99.8-600 t/ha (converted)	Reduced germination and seedling establishment; Decreased biomass and yield; Phytotoxic effects due to salinity and nutrient imbalance	Elevated soil EC (salinity stress); Accumulation of heavy metals/ micronutrients (Zn, Mn, Cu, B); Nutrient antagonism and impaired soil quality	Reduced microbial diversity and community stability; Dominance of opportunistic taxa (e.g., Acinetobacter) • Increased CO ₂ and N ₂ O emissions, resembling fresh manure effects

*Note.**Application ranges vary depending on soil type, crop species, and frass composition

Potential Research Gaps

There is a lack of standardisation in frass composition and quality to produce the nutrient frass. For example, large variability in larvae feedstock (e.g., brewery waste, kitchen scraps, animal manure) leads to inconsistent frass nutrient profiles (e.g., N, P, K, micronutrients) and physicochemical properties (pH, EC, C: N ratio). There is no universally accepted quality standard or classification system for BSFL frass (e.g., based on NPK levels, heavy metals, and pathogen load). Research rarely evaluates microbial safety, persistence of antibiotics or pathogens, or potential allelopathic compounds. Future research should focus on defining clear standards for BSFL frass composition, nutrient content labelling, and safety testing protocols.

There is also a limited understanding of long-term soil and environmental effects. Most studies are short-term and conducted under controlled conditions (greenhouse, pot trials), with few long-term or multi-season field experiments. Soil health over time, such as microbial community stability, nutrient leaching, and buildup of salts or heavy metals, is largely unexamined. Greenhouse gas (GHG) emissions from frass-amended soils (e.g., CO₂, N₂O, CH₄) are poorly studied in terms of across different climatic zones and soil types. Longitudinal studies are needed to monitor the cumulative impacts of repeated frass application on soil ecology, crop health, and environmental emissions.

Moreover, there are also no studies on interaction with other inputs such as fertilisers, biochar, and compost. Synergistic or antagonistic effects of BSFL frass when combined with other organic or inorganic fertilisers are underexplored. Limited investigation into how frass performs in integrated nutrient management systems, or as a partial replacement for synthetic fertilisers. Studies should assess optimised frass-blended fertilisation strategies for maximising plant response while reducing input costs and environmental burden.

Furthermore, crop- and soil-specific dosing guidelines are incomplete. While some studies report optimum rates, most findings are context-specific and not scalable or generalisable. Little guidance exists for applying BSFL frass across different soil textures, pH levels, salinity conditions, or crop developmental stages. A decision-support framework is needed to guide farmers in determining safe and effective frass application rates tailored to local agroecological conditions.

Additionally, plant-soil-microbe interactions are underexplored. Microbial shifts in frass application rate, such as both beneficial and detrimental, are observed. Yet, the mechanistic insights into how frass application rate affects plant-microbe symbioses, root microbiome recruitment, and disease suppression or promotion are lacking. Few studies explore frass effects on mycorrhizal fungi, soil enzymes, or microbial functional traits critical for nutrient cycling and soil structure. Metagenomic and metabolomic tools should be studied to unravel how frass influences plant-soil-microbe interactions across different settings.

Plus, economic and agronomic feasibility in real-world farming systems is limited by the application rate of frass. Most studies are not linked to cost-benefit analyses, labour and resource requirements, or logistics of sourcing and applying frass at scale. Farmer adoption, especially in low-income or smallholder settings, is poorly understood. Socioeconomic studies and on-farm participatory research are essential to assess the feasibility, acceptance, and scalability of BSFL frass in different agricultural contexts.

CONCLUSION

Based on the 16 selected studies, BSFL frass has the potential to become a sustainable fertiliser. Direct soil incorporation is most common and effective, while N-based or combined applications improve nutrient use efficiency; pot and incubation studies clarify mechanisms but are less field-relevant. However, there is no specific application rate. Moderate rates via incorporation or combined fertilisation enhance plant growth and soil fertility; high rates can cause salinity, nutrient imbalance, and reduced productivity. The studied application rate ranges from 10 to 45 t/ha, which is too high to apply in practice. Yet, more future research is needed to move beyond proof-of-concept and address standardisation, scalability, environmental safety, and long-term ecosystem effects. Interdisciplinary efforts involving agronomy, microbiology, soil science, and economics are essential to bridge these gaps and integrate BSFL frass into regenerative agriculture models.

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REFERENCES

- Abd El-Hack, M. E., Shafi, M. E., Alghamdi, W. Y., Abdelnour, S. A., Shehata, A. M., Noreldin, A. E., Ashour, E. A., Swelum, A. A., Al-Sagan, A. A., Alkhateeb, M., Taha, A. E., Abdel-Moneim, A.-M. E., Tufarelli, V., & Ragni, M. (2020). Black soldier fly (*Hermetia illucens*) meal as a promising feed ingredient for poultry: A comprehensive review. *Agriculture*, 10(8), Article 339. <https://doi.org/10.3390/agriculture10080339>
- Abd Manan, F., Yeoh, Y.-K., Chai, T.-T., & Wong, F.-C. (2024). Unlocking the potential of black soldier fly frass as a sustainable organic fertiliser: A review of recent studies. *Journal of Environmental Management*, 367, Article 121997. <https://doi.org/10.1016/j.jenvman.2024.121997>
- Ambrosini, A., de Souza, R., & Passaglia, L. M. P. (2016). Ecological role of bacterial inoculants and their potential impact on soil microbial diversity. *Plant and Soil*, 400(1), 193-207. <https://doi.org/10.1007/s11104-015-2727-7>
- Amorim, H. C. S., Ashworth, A. J., Arsi, K., Rojas, M. G., Morales-Ramos, J. A., Donoghue, A., & Robinson, K. (2024). Insect frass composition and potential use as an organic fertiliser in circular economies. *Journal of Economic Entomology*, 117(4), 1261-1268. <https://doi.org/10.1093/jee/toad234>

- Ashworth, A. J., Amorim, H. C. S., Drescher, G. L., Moore, P. A., Rojas, M. G., Morales-Ramos, J., & Donoghue, A. M. (2025). Insect frass fertiliser as soil amendment for improved forage and soil health in circular systems. *Scientific Reports*, 15(1), Article 3024. <https://doi.org/10.1038/s41598-025-87075-8>
- Bashir, O., Ali, T., Baba, Z. A., Rather, G. H., Bangroo, S. A., Mukhtar, S. D., Naik, N., Mohiuddin, R., Bharati, V., & Bhat, R. A. (2021). Soil organic matter and its impact on soil properties and nutrient status. In G. H. Dar, R. A. Bhat, M. A. Mehmood, & K. R. Hakeem (Eds.), *Microbiota and biofertilisers, Vol 2: Ecofriendly tools for reclamation of degraded soil environs* (pp. 129-159). Springer International Publishing. https://doi.org/10.1007/978-3-030-61010-4_7
- Beesigamukama, D., Mochoge, B., Korir, N. K., Fiaboe, K. K. M., Nakimbugwe, D., Khamis, F. M., Subramanian, S., Dubois, T., Musyoka, M. W., Ekesi, S., Kelemu, S., & Tanga, C. M. (2020). Exploring black soldier fly frass as novel fertiliser for improved growth, yield, and nitrogen use efficiency of maize under field conditions. *Frontiers in Plant Science*, 11, Article 574592. <https://doi.org/10.3389/fpls.2020.574592>
- Beesigamukama, D., Mochoge, B., Korir, N., Musyoka, M. W., Fiaboe, K. K. M., Nakimbugwe, D., Khamis, F. M., Subramanian, S., Dubois, T., Ekesi, S., & Tanga, C. M. (2020). Nitrogen fertiliser equivalence of black soldier fly frass fertiliser and synchrony of nitrogen mineralisation for maize production. *Agronomy*, 10(9), Article 1395. <https://doi.org/10.3390/agronomy10091395>
- Borkent, S., & Hodge, S. (2021). Glasshouse evaluation of the black soldier fly waste product HexaFrass™ as an organic fertiliser. *Insects*, 12(11), Article 977. <https://doi.org/10.3390/insects12110977>
- Buckley, S., Coleman, J., Davison, I., Khan, K. S., Zamora, J., Malick, S., Morley, D., Pollard, D., Ashcroft, T., Popovic, C., & Sayers, J. (2009). The educational effects of portfolios on undergraduate student learning: A Best Evidence Medical Education (BEME) systematic review. BEME Guide No. 11. *Medical Teacher*, 31(4), 282-298. <https://doi.org/10.1080/01421590902889897>
- Carroll, A., Fitzpatrick, M., & Hodge, S. (2023). The effects of two organic soil amendments, biochar and insect frass fertiliser, on shoot growth of cereal seedlings. *Plants*, 12(5), Article 1071. <https://doi.org/10.3390/plants12051071>
- Chavez, M. Y., Uchanski, M., & Tomberlin, J. K. (2023). Impacts of black soldier fly, (Diptera: Stratiomyidae) larval frass on tomato production. *Journal of Economic Entomology*, 116(5), 1490-1495. <https://doi.org/10.1093/jee/toad150>
- Chele, K. H., Tinte, M. M., Piater, L. A., Dubery, I. A., & Tugizimana, F. (2021). Soil salinity, a serious environmental issue and plant responses: A metabolomics perspective. *Metabolites*, 11(11), Article 724. <https://doi.org/10.3390/metabo11110724>
- Chen, Q., Song, Y., An, Y., Lu, Y., & Zhong, G. (2024). Soil microorganisms: Their role in enhancing crop nutrition and health. *Diversity*, 16(12), Article 734. <https://doi.org/10.3390/d16120734>
- Chiam, Z., Lee, J. T. E., Tan, J. K. N., Song, S., Arora, S., Tong, Y. W., & Tan, H. T. W. (2021). Evaluating the potential of okara-derived black soldier fly larval frass as a soil amendment. *Journal of Environmental Management*, 286, Article 112163. <https://doi.org/10.1016/j.jenvman.2021.112163>
- Culas, R. J., Anwar, M. R., & Maraseni, T. N. (2025). A framework for evaluating benefits of organic fertiliser use in agriculture. *Journal of Agriculture and Food Research*, 19, Article 101576. <https://doi.org/10.1016/j.jafr.2024.101576>

- Gurung, S. K., Mickan, B. S., Middleton, J. A., Singh, P. K., Jenkins, S. N., Rengel, Z., Siddique, K. H. M., & Solaiman, Z. M. (2024). Manure-derived black soldier fly frass enhanced the growth of chilli plants (*Capsicum annuum* L.) and altered rhizosphere bacterial community. *Applied Soil Ecology*, 202, Article 105605. <https://doi.org/10.1016/j.apsoil.2024.105605>
- Havlin, J. L. (2020). Soil: Fertility and nutrient management. In *Landscape and Land Capacity* (2nd ed.). CRC Press. <https://doi.org/10.1201/9780429445552-34>
- Huang, Z., Xu, S., Zheng, N., Yin, X., Yang, Y., & Yao, H. (2024). Salty bio-converted organic fertiliser modulates soil greenhouse gas emissions. *Journal of Cleaner Production*, 445, Article 141192. <https://doi.org/10.1016/j.jclepro.2024.141192>
- Lomonaco, G., Franco, A., De Smet, J., Scieuzo, C., Salvia, R., & Falabella, P. (2024). Larval frass of *Hermetia illucens* as organic fertiliser: Composition and beneficial effects on different crops. *Insects*, 15(4), Article 293. <https://doi.org/10.3390/insects15040293>
- Malheiro, C., Prodana, M., Patinha, C., Morgado, R. G., & Loureiro, S. (2024). Insect frass from upcycling vegetable by-products with cereals: Effects on the soil properties, plant development and soil invertebrate fitness. *Journal of Environmental Management*, 372, Article 123234. <https://doi.org/10.1016/j.jenvman.2024.123234>
- Maron, P.-A., Sarr, A., Kaisermann, A., Lévêque, J., Mathieu, O., Guigue, J., Karimi, B., Bernard, L., Dequiedt, S., Terrat, S., Chabbi, A., & Ranjard, L. (2018). High microbial diversity promotes soil ecosystem functioning. *Applied and Environmental Microbiology*, 84(9), Article e02738-17. <https://doi.org/10.1128/AEM.02738-17>
- Meena, M., Swapnil, P., Zehra, A., Aamir, M., Dubey, M. K., Goutam, J., & Upadhyay, R. S. (2017). Beneficial microbes for disease suppression and plant growth promotion. In D. P. Singh, H. B. Singh, & R. Prabha (Eds.), *Plant-microbe interactions in agro-ecological perspectives: Volume 2: Microbial interactions and agro-ecological impacts* (pp. 395-432). Springer. https://doi.org/10.1007/978-981-10-6593-4_16
- Menino, R., Felizes, F., Castelo-Branco, M. A., Fareleira, P., Moreira, O., Nunes, R., & Murta, D. (2021). Agricultural value of black soldier fly larvae frass as organic fertiliser on ryegrass. *Heliyon*, 7(1), Article e05855. <https://doi.org/10.1016/j.heliyon.2020.e05855>
- Moher, D., Liberati, A., Tetzlaff, J., & Altman, D. G. (2009). Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *BMJ*, 339, Article b2535. <https://doi.org/10.1136/bmj.b2535>
- Mostafaie, A., Silva, A. R. R., N. Pinto, J., Prodana, M., Lopes, I. G., Murta, D., Brooks, B. W., Loureiro, S., & Cardoso, D. N. (2025). Towards circularity for agro-waste: Minimal soil hazards of olive pomace bioconverted frass by insect larvae as an organic fertiliser. *Journal of Environmental Management*, 375, Article 124151. <https://doi.org/10.1016/j.jenvman.2025.124151>
- Oteri, M., Di Rosa, A. R., Lo Presti, V., Giarratana, F., Toscano, G., & Chiofalo, B. (2021). Black soldier fly larvae meal as alternative to fish meal for aquaculture feed. *Sustainability*, 13(10), Article 5447. <https://doi.org/10.3390/su13105447>
- Poveda, J. (2021). Insect frass in the development of sustainable agriculture. A review. *Agronomy for Sustainable Development*, 41(1), Article 5. <https://doi.org/10.1007/s13593-020-00656-x>

- Putri, H. H., Lakitan, B., Negara, Z. P., & Muda, S. A. (2023). Growth of red amaranth (*Amaranthus cruentus* L.) cultivated on soil-based substrate amended with a residue of the black soldier fly larvae containing heavy metals. *HAYATI Journal of Biosciences*, 30(2), 302-312. Scopus. <https://doi.org/10.4308/hjb.30.2.302-312>
- Quigley, J. D., Zontini, A., Schroeder, G. F., Roman-Garcia, Y., Houbiers, L., & Bach, A. (2025). Nutritional value of black soldier fly larvae oil in calf milk replacers. *Journal of Dairy Science*, 108(3), 2481-2488. <https://doi.org/10.3168/jds.2024-25666>
- Rodgers, E., Nicolson, E., Lauder, S., & Hodge, S. (2024). Response of pasture grasses to organic fertiliser produced from black soldier fly frass. *Plants*, 13(7), Article 943. <https://doi.org/10.3390/plants13070943>
- Rummel, P. S., Beule, L., Hemkemeyer, M., Schwalb, S. A., & Wichern, F. (2021). Black soldier fly diet impacts soil greenhouse gas emissions from frass applied as fertiliser. *Frontiers in Sustainable Food Systems*, 5, Article 709993. <https://doi.org/10.3389/fsufs.2021.709993>
- Sabina, R., Paul, J., Sharma, S., & Hussain, N. (2025). Synthetic nitrogen fertiliser pollution: Global concerns and sustainable mitigating approaches. In N. Hussain, C.-Y. Hung, & L. Wang (Eds.), *Agricultural nutrient pollution and climate change: Challenges and opportunities* (pp. 57-101). Springer Nature Switzerland. https://doi.org/10.1007/978-3-031-80912-5_3
- Salomon, M. J., Cavagnaro, T. R., & Burton, R. A. (2025). Potential of black soldier fly larvae frass (BSFL) as a novel fertiliser: Impacts on tomato growth, nutrient uptake, and mycorrhizal formation. *Plant and Soil*, 501(1-2), 115-129. <https://doi.org/10.1007/s11104-024-07187-4>
- Sarkar, D., & Baishya, L. K. (2017). Nutrient use efficiency. In M. Naeem, A. A. Ansari, & S. S. Gill (Eds.), *Essential plant nutrients: Uptake, use efficiency, and management* (pp. 119-146). Springer International Publishing. https://doi.org/10.1007/978-3-319-58841-4_6
- Sawinska, Z., Radzikowska-Kujawska, D., Kowalczewski, P. Ł., Grzanka, M., Sobiech, Ł., Skrzypczak, G., Drożdżyńska, A., Ślachciński, M., & Świtek, S. (2024). *Hermetia illucens* frass fertilisation: A novel approach for enhancing lettuce resilience and photosynthetic efficiency under drought stress conditions. *Applied Sciences*, 14(6), Article 2386. <https://doi.org/10.3390/app14062386>
- Watson, C., Schlösser, C., Vögerl, J., & Wichern, F. (2021). Excellent excrement? Frass impacts on a soil's microbial community, processes and metal bioavailability. *Applied Soil Ecology*, 168, Article 104110. <https://doi.org/10.1016/j.apsoil.2021.104110>
- Wu, N., Yu, X., Liang, J., Mao, Z., Ma, Y., Wang, Z., Wang, X., Liu, X., & Xu, X. (2023). A full recycling chain of food waste with straw addition mediated by black soldier fly larvae: Focus on fresh frass quality, secondary composting, and its fertilising effect on maize. *Science of The Total Environment*, 885, Article 163386. <https://doi.org/10.1016/j.scitotenv.2023.163386>

SUPPLEMENTARY MATERIALS

Supplementary Table S1
Databases searched and records retrieved

Database	Search Date	Records Identified
PubMed	15 May 2025	7
ScienceDirect	15 May 2025	52
Scopus	15 May 2025	37
Emerald Insight	15 May 2025	0
Total		96

Supplementary Table S2
Detailed search strategies used in each database

Database	Search String
PubMed	("Hermetia illucens" OR "black soldier fly" OR BSF) AND ("black soldier fly larvae frass" OR "BSFL frass" OR "black soldier fly frass" OR "BSF frass" OR "insect frass") AND ("application rate" OR "soil to frass ratio" OR "frass to soil ratio" OR "frass percentage" OR "frass-soil mix*")
ScienceDirect - Search 1	("black soldier fly") AND ("black soldier fly larvae frass" OR "black soldier fly frass" OR "bsf frass" OR "insect frass") AND ("application rate" OR "soil to frass ratio" OR "frass to soil ratio" OR "frass percentage")
ScienceDirect - Search 2	("Hermetia Illucens" OR "black soldier fly" OR bsf) AND ("black soldier fly larvae frass" OR "black soldier fly frass" OR "insect frass") AND ("application rate" OR "frass-soil mixture")
Scopus	("Hermetia Illucens" OR "black soldier fly" OR bsf) AND ("black soldier fly larvae frass" OR "bsfl frass" OR "black soldier fly frass" OR "bsf frass" OR "insect frass") AND ("application rate" OR "frass to soil ratio" OR "frass percentage" OR "frass-soil mix*")
Emerald Insight	("Hermetia Illucens" OR "black soldier fly" OR bsf) AND ("black soldier fly larvae frass" OR "bsfl frass" OR "black soldier fly frass" OR "bsf frass" OR "insect frass") AND ("application rate" OR "soil to frass ratio" OR "frass to soil ratio" OR "frass percentage" OR "frass-soil mix*")

Note. The asterisk (*) indicates truncation in the search strategy, allowing retrieval of records containing different endings of the root word. For example, mix* includes mix, mixed, mixing, and mixture

Influence of NPK Compound Fertiliser Fertigation on Guava Fruit Yield and Quality in Nghe An Province, Vietnam

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ABSTRACT

Nitrogen (N), phosphorus (P), and potassium are the most important macronutrients for plants, particularly for guava trees, which are in the flowering, fruiting stage, and fruit development. The objective of this research was to determine the impact of levels of NPK compound Fertiliser applied via irrigation system on guava fruit yield and quality. The experiment was set up in a randomised complete block design with seven treatments and three replications. The NPK compound Fertiliser (19-19-19 + 3CaO + Te) was applied through drip irrigation according to six levels of treatment (Fertiliser amounts of 400, 600, 800, 1000, 1200, and 1400 g/plant/year) and control. The number of flowers, yield components, and quality parameters were measured. The results demonstrated that fertigation with 1000 g/plant/year of NPK compound Fertiliser led to significant increases of

the fruits set (168.5 fruits/plant), haverst fruits (91.8 fruits/plant), fruit weight (368.4 g), yield (22.5 tons/ha), pulp thickness (29.1 mm), core thickness (44.9 mm), fruit diameter (70.9 mm), fruit flesh weight ratio (76.6 %), and total soluble solids (9.8 %). The results can be used to develop technical standards to produce the best guava yield and quality under the ecological situation of Nghe An Province.

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INTRODUCTION

Guava (*Psidium guajava* L.) is one of the most popular fruit plants in Vietnam and the world. Guava fruit has high nutritional value with high vitamin C content (75-260 mg/100 g) and is a good source of thiamine (0.03-0.07 mg/100 g), riboflavin (0.02-0.04 mg/100 g), phosphorus (22.5-40 mg/100 g), calcium (10-30 mg/100 g), iron (20-25 mg/100 g), pectin (0.5-1.8%) and many antioxidants that help control blood pressure (Shukla et al., 2009). The Taiwanese pear guava was introduced to Vietnam last year. The guava is easy to grow and can be grown in various climatic and soil conditions in Vietnam. This variety has high yield and quality and produces 3-4 crops every year.

Guava plants start blooming and giving fruit at the same time; hence, it is important to meet their nutritional demands. Nitrogen (N), phosphorus (P), potassium (K) (Nutrients are called macronutrients), micronutrients, and secondary nutrients are essential for guava. Lack of these nutrients during the flowering period and fruit formation will affect yield and fruit quality. Increasing the level of NPK Fertiliser resulted in an increase in fruit set, fruit numbers/tree, and fruit weight (Khan et al., 2018). Successive increases in the rate of NPK fertilisation from 65:30:30 to 135:60:60 g/tree increased the number of fruits, fruit weight, diameter and fruit yield. The best fruit yield and fruit quality were obtained by applying NPK Fertiliser to guava plants at 135:60:60 g/plant (Thirupathi et al., 2016). Using inorganic, organic, and micronutrient Fertilisers resulted in significantly higher flowering, diameter, length, number of fruits, and fruit yield (Gupta et al., 2019). Guava plants' nutrient demands are influenced by both soil fertility (ability to provide nutrients) and plant population. When guava is grown at a planting distance of 2×1 m, the recommended dosage for maximum yield and fruit quality is 400 g N, 200 g P_2O_5 , and 200 g K_2O (Guleria & Singh, 2023). There is a lower requirement for each plant when the planting density is 3×3 m (300 g N, 150 g P_2O_5 , and 150 g K_2O) (Chavan et al., 2020). Applying Fertilisers through irrigation systems will ensure sufficient water and Fertiliser supplies to guava trees while reducing Fertiliser and labour costs. Drip irrigation significantly affects yield and guava fruit quality while reducing the cost of Fertilisers (Challa et al., 2021). Fertiliser application through irrigation using soluble Fertiliser decreased 25% of the Fertiliser used while maintaining guava yield and quality (Janaki et al., 2020).

One of the wards in Nghe An province with a large guava production is Tay Hieu. Applying soluble macronutrients, micronutrients, and trace elements through the irrigation system supplies water and nutrients constantly to ensure the continuous flowering and bearing of guava plants. Although there have been many studies on guava Fertilisers in Vietnam, the studies on the use of soluble complex Fertilisers via the irrigation system are scarce. Thus, the purpose of this study is to identify the optimal dose of NPK 19-19-19 + 3CaO + Te complex Fertiliser applied through a drip irrigation system to demonstrate high yield and quality production of the Taiwanese pear guava variety in the ecosystem of Nghe An province.

MATERIALS AND METHODS

Treatments and Experimental Design

The study was performed from October 2023 to October 2024 in the guava garden of Tay Hieu ward, Nghe An province (105.28°N, 19.10°E). The experiment was performed on Vetis-Acric Ferralsol with soil with the chemical properties of the soil in Table 1, and the temperature, rainfall, and humidity during the experiment in Figure 1.

The reproductive success and yield of guava are heavily controlled by environmental factors, especially temperature and soil characteristics, which in turn affect the response to NPK compound Fertiliser on drip irrigation. This work combines the temperature inferred

Table 1
Soil chemical properties at the study site (0 - 20 cm depth)

Properties	Values	Properties	Values
pH (1: 2.5 soil water suspension)	4.71	DTPA-extractable Mn (mg/kg)	192
ECe (mS/cm)	0.283	DTPA-extractable Fe (mg/kg)	134
Organic Carbon (%)	0.7	DTPA-extractable Cu (mg/kg)	2.83
Total N (%)	0.12	Soil Texture	
Available P (mg/kg)	71.56	Sand (%)	32.0
Exchangeable K (mg/kg)	65.54	Silt (%)	23.0
Exchangeable Ca (mg/kg)	359	Clay (%)	45.0
Exchangeable Mg (mg/kg)	103	Soil classification	Veti-Acric Ferralsol
DTPA-extractable Zn (mg/kg)	3.56		

Note. Soil samples were analysed at the Centre for Environmental Analysis and Technology Transfer (CEATT), Vietnam Academy of Agricultural Sciences (VAAS), Hanoi, Vietnam

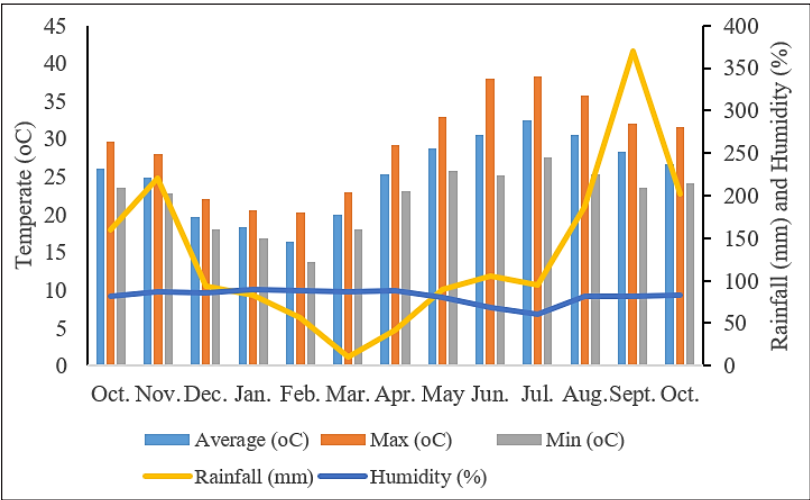


Figure 1. Temperature, rainfall and humidity during the study

from weather reports during the study conducted in Nghe An Province, Vietnam, and the estimated soil chemical properties inferred from the study's context (Figure 1 and Table 1). Temperatures optimal for flowering and fruiting (23-28 °C) decrease the reproductive potential of guava, which stunts the growing process at temperatures below 15.5 °C and substantially halts growth at -3 °C (Fischer & Melgarejo, 2021; Haokip et al., 2020).

Variations in temperature over time from suboptimal winter temperatures (Phase 2) to optimal summer temperatures (Phases 3-4) corresponded well with fruit set changes, respectively, lowest in Phase 2 and highest in Phases 3-4. There were no freezes (-3 °C), preventing severe damage, but Phase 2's lower temperatures (15-20 °C) likely caused growth inhibition, consistent with the 15.5 °C minimum metabolic temperature for guava (Haokip et al., 2020).

The experiment was conducted on a Taiwanese pear guava variety grafted onto wild guava rootstock. The plants were planted in 2018 with a planting density of 666 plants per hectare (3.0 m × 5.0 m). All of the orchards were managed in a standard way, including water stress management with drip irrigation, pruning, mowing, fruit wrapping, and disease control.

In this study, we applied a compound Fertiliser, NPK 19 (N)-19 (P) -19 (K) + 3CaO + Te from the Israeli company ICL. The Fertiliser has a nutritional composition of: Macronutrients: 19% N, 19% P₂O₅, 19% K₂O; secondary nutrients: 3% CaO; micronutrients (ppm): Fe: 400, Mn: 200, Zn: 100, Cu: 50, Mo: 50, Bo: 360. The second Fertiliser used was a base Fertiliser of composted cow manure from the Green Earth product line by Nong Sinh company.

A micro-irrigation system by Netafim, Israel (Dripnet PC 16150) was used for this study. It has a double line of drip heads, with a distance of 1 meter from the plant base. Emitters are 0.5 meters apart and have a flow rate of 1.0 litres/hour. With plants spaced 3 meters apart, 6 meters of irrigation line is dedicated to each plant (12 drip heads). Thus, each plant receives 12 litres/hour (12 drip heads × 1.0 liter/hour/drip head).

The experiment was conducted in a randomised complete block design (RCB) with seven irrigation treatments and 3 replications. Each of the replications had 5 guava plants of similar age and canopy that were selected for data recording. In all, a total of 105 guava plants were used with a total plot size of 1,575 m². Details of experimental treatments are shown in Table 2.

Control (T7) follows local farming practices. The total Fertiliser applied per plant for each year is 940 g of N, 480 g of P₂O₅, and 840 g of K₂O. Fertilising for flowering: For each plant, apply 300 g of NPK 16-16-8 + Te (Cu: 100 ppm, Zn: 100 ppm) and 100 g of Urea. It must be applied just after pruning (October, 2023) to induce flowering. Fertilising for fruit: For each plant, apply 200 g of NPK 16-16-8 + Te, 100 g of Urea, and 100 g of KCl. Apply 1.5 months after the flowering fertilisation. Make the application every 15

Table 2
Fertiliser rates for experimental treatments

Treatment	Fertiliser Type	Amount of Fertiliser (g/plant /year)	Amount of Fertiliser (kg/ha/year)	Number of Irrigations/year	Amount of Fertiliser (g/15 plants/time)	Nutrient concentration (ppm)
T1	NPK 19-19-19+3CaO+TE	400	266.4	28	214.3	214.3
T2	NPK 19-19-19+3CaO+TE	600	399.6	28	321.4	321.4
T3	NPK 19-19-19+3CaO+TE	800	532.8	28	428.6	428.6
T4	NPK 19-19-19+3CaO+TE	1,000	666.0	28	535.7	535.7
T5	NPK 19-19-19+3CaO+TE	1,200	799.2	28	642.9	642.9
T6	NPK 19-19-19+3CaO+TE	1,400	932.4	28	750.0	750.0
T7 (Control)	Farmer practice					

days and the same weight for 10 applications during the experiment. Fertilising method: Excavate a trench around the canopy volume, 0.7 m from the trunk. Place Fertiliser in the groove and fill with soil.

Basal fertilisation: In October 2023, all formulas were fertilised with 22 kg of composted cow manure and 1 kg of lime powder per plant annually.

Pruning and shaping: Prune any diseased fruits and branches. Also, remove branches with excessive fruit, small branches, those near the ground, and excess branches in the canopy.

Bagging: When the guava fruit is 2.5-3 cm in diameter, wrap it with a foam and plastic bag with holes.

Water calculation for guava plants: all formulas were under the same drip irrigation system. The use of water was the same in all formulas, calculated and presented in Table 3.

Table 3
Calculation of water quantity for guava plants during the experimental period

Month. Year	ETo	KC	Etc	Efr	IWN1	IWN2	IWN3
Oct. 2023	110.2	0.6	66.1	63.0	3.1	31.2	4.9
Nov.2023	106.4	0.7	74.5	68.5	6.0	59.8	9.4
Dec. 2023	93.2	0.8	74.6	67.6	7.0	69.6	11.0
Jan. 2024	93.9	0.9	84.5	8.0	76.5	765.1	120.5
Feb. 2024	100.9	1.0	100.9	4.0	96.9	969.0	152.6
Mar. 2024	123.4	1.1	135.7	4.0	131.7	1317.4	207.5
Apr. 2024	133.0	1.2	159.6	2.0	157.6	1576.0	248.2
May. 2024	161.9	1.3	210.5	2.0	208.5	2084.7	328.3
Jun. 2024	171.2	1.3	222.6	5.0	217.6	2175.6	342.7
Jul. 2024	175.6	1.3	228.3	5.0	223.3	2232.8	351.7
Aug. 2024	133.2	1.2	159.8	48.8	111.0	1110.4	174.9
Sept. 2024	110.3	0.8	88.2	60.4	27.8	278.4	43.8
Oct. 2024	102.8	0.6	61.7	60.5	1.2	11.8	1.9

Note. ETo: Evapotranspiration (mm/month). KC: Crop coefficient. ETo: plant-water requirement (mm/month). Efr: Effective rainfall (mm/month). IWN1: Irrigation water need (mm/month). Irrigation water need: IWN2 (m³/month/ha). Irrigation water need: IWN3 (m³/month/1575.5 m²)

Data Collection and Analysis

Five plants in each replication were used to collect data. So, 15 plants were surveyed per treatment. A total of 105 plants were used.

Number of Flowers per Plant

In each plant, four major branches in the canopy outer part were marked to monitor and record the number of flowers per plant during flowering periods

Number of Fruits per Plant

Count the number of fruits per plant in each harvest.

Fruit Set

The calculation for fruit set can be found in Equation 1.

$$\text{Fruit set (\%)} = \frac{\text{Number of fruits per plant}}{\text{Number of flowers per plant}} \times 100 \quad [1]$$

Average Yield per Plant (kg/plant)

Weigh all commercial fruits at each harvest and calculate the total during the experimental period.

Average Yield per ha (ton/ha)

Average yield (kg/plant) $\times$ plant density.

Fruit Drop

The calculation for fruit drop can be found in Equation 2.

$$\text{Fruit drop (\%)} = \frac{\text{Number of dropped fruits per plant}}{\text{Total number of fruits per plant}} \times 100 \quad [2]$$

Fruit Indicators

Select and harvest ripe fruit. The sample size for measurement is 2 fruits per plant, which totals 10 fruits per replication and 30 fruits per treatment

Average Fruit Weight (grams)

Weigh the selected fruit in each treatment using an electronic scale with an accuracy of ± 0.01 grams. Pulp thickness (mm): Cut the fruit lengthwise, then measure from the skin to the part adjacent to the fruit pulp and calculate the average value.

Fruit Flesh Weight Ratio

The calculation for fruit flesh weight ratio can be found in Equation 3.

$$\text{Fruit flesh weight ratio (\%)} = \frac{\text{Fruit flesh weight}}{\text{Fruit weight}} \times 100 \quad [3]$$

Total soluble solids (TSS) (%)

The guava fruits were selected, juice was extracted and measured using a portable refractometer AS ONE (Japan).

Statistical Analysis

All data were analysed using IRRISTAT version 5.0. One-way analysis of variance (ANOVA) was conducted to evaluate treatment effects, and mean differences were separated using the Least Significant Difference (LSD) test at a 5% significance level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Flowering and Fruit Setting of Guava Plants

Flowering and fruit set performance are vital factors affecting productivity in fruit plants, including guava plants. Several factors, such as climate, management practices, and, importantly, macronutrient supply, affect the flowering and fruit set processes in guava plants (Rangare et al., 2025). Four phases of the flowering and fruiting process were detected in the guava plants: Phase 1 (November-December 2023), Phase 2 (January-February 2024), Phase 3 (April-May 2024), and Phase 4 (June-July 2024). The Least Significant Difference (LSD) test ($P < 0.05$) and low coefficient of variation (CV%, 1.1-8.9%) ensure statistical consistency and the precision of the data. Rates, which may be due to the chemically unbalanced NPK Fertilisers, are conducive to leaf and shoot development

Table 4 shows the effect of NPK Fertiliser on guava growth during four phases of flowering, about flowers/plant, fruits set/plant, and fruit set (%). During the first phase, the number of flowers per plant ranged from 36.9 to 50.2, and the number of fruits set per plant ranged from 17.4 to 26.2. The highest number of flowers and fruits was set with statistically significant differences to other Fertiliser rates, and the control was produced when 1000 g per plant of NPK compound Fertiliser was applied. The fruit set in this phase was between 44.4% and 52.2%. The application rates of NPK compound Fertilisers of 400, 750, and 1000 g per plant showed a high fruit set; nevertheless, above 1000 g, the trend of fruit set was downwards. These results indicate that higher rates of N in the NPK compound Fertilisers make plants focus on their leaf and stem growth rather than flowering and fruit set. This result is similar to that of Kumari et al. (2018), who showed that higher levels of N from 600 to 800 g per plant tended to reduce the fruit set. The number of flowers in the second phase varied between 48.9 and 57.6 flowers per plant. Despite a greater number of flowers compared to the first phase, fewer fruits were set, and percentages of fruit set ranged from 17.3 to 24.5 fruits per plant and 33.9% to 42.5%, respectively (Table 4). The dripping rain and low temperatures of this period may play a role in the low fruit set (Table 3). These effects have also been reported in tropical and subtropical fruit species like mango, guava,

Table 4
Effect of NPK compound Fertiliser application through irrigation system on flowering and fruit setting of guava plants

Fertiliser Rate (g/plant)	Phase 1 (From November to December 2023)			Phase 2 (From January to February 2024)			Phase 3 (From April to May 2024)			Phase 4 (From June to July 2024)		
	Flowers/ Plant	Fruit Set/ Plant	Fruit Set (%)	Flowers/ Plant	Fruit Set/ Plant	Fruit Set (%)	Flowers/ Plant	Fruit Set/ Plant	Fruit Set (%)	Flowers/ Plant	Fruit Set/ Plant	Fruit Set (%)
400	41.9 ^b	21.7 ^{bc}	51.8 ^a	56.6 ^a	20.5 ^{bc}	36.2 ^{bc}	57.5 ^b	51.4 ^b	89.3 ^{ab}	53.1 ^b	46.1 ^b	86.7 ^{ab}
600	43.5 ^b	22.6 ^b	52.1 ^a	59.5 ^a	21.8 ^{ab}	36.6 ^{bc}	58.6 ^b	53.3 ^b	90.9 ^a	56.6 ^b	49.9 ^b	88.2 ^a
800	44.7 ^b	23.1 ^b	51.8 ^a	53.1 ^{ab}	21.6 ^{ab}	38.8 ^{ab}	58.9 ^b	52.3 ^b	89.0 ^{ab}	57.6 ^b	50.1 ^b	87.1 ^a
1000	50.2 ^a	26.2 ^a	52.2 ^a	57.6 ^a	24.5 ^a	42.5 ^a	63.5 ^a	59.3 ^a	93.4 ^a	65.3 ^a	58.5 ^a	89.6 ^a
1200	42.5 ^b	19.7 ^{cd}	46.5 ^b	54.8 ^{ab}	18.6 ^{bc}	33.9 ^c	51.3 ^c	43.9 ^c	85.5 ^{bc}	44.0 ^c	36.8 ^c	83.6 ^{bc}
1400	42.8 ^b	19.0 ^d	44.4 ^b	54.0 ^{ab}	19.8 ^{bc}	36.7 ^{bc}	48.9 ^c	43.9 ^c	89.8 ^{ab}	44.5 ^c	36.5 ^c	82.0 ^c
Control	36.9 ^c	17.4 ^d	47.4 ^b	48.9 ^b	17.3 ^c	35.4 ^{bc}	41.5 ^d	34.6 ^d	83.3 ^c	46.7 ^c	38.0 ^c	81.3 ^c
LSD _{0.05}	4.4	2.3	3.5	6.4	3.7	4.2	3.3	3.4	4.3	6.2	5	3.1
CV %	5.8	6.3	4.1	5.3	5.9	6.8	3.4	3.9	2.8	6.7	6.3	2.0

Note. Different letters within columns indicate significant differences at $P \leq 0.05$

and lychee, where low temperature during flowering results in disrupted flowering, high flowering drop, and fruit set (Haokip et al., 2020).

In the third phase, the number of flowers in plants ranged from 41.5 to 63.5, with no significant difference in comparison to the second phase. But the growth in fruit set was encouraging. The highest numbers of flowers and fruits set per plant were at an NPK application rate of 1000 g, rating the peak, and showing a statistically significant difference when compared to other rates and the control. Stunningly, the fruit set increased from 83.3% to 93.4%, with 1000 g, claiming the highest rate of fruit set. The more favourable climatic conditions at this phase, with an average temperature of 28.7 °C, as well as day maximum and minimum of 32.9 °C and 25.7 °C (Table 3), respectively, certainly contributed to such a remarkable fruit setting percentage. Indeed, the work of Fischer and Melgarejo (2021) highlights this observation, showing that guava plants have optimum growth between 15 °C and 30 °C; beyond this, flower loss (drop) and consequent reduction in fruit set increase.

In the fourth phase, the phenomenon continued to amaze, with the number of flowers and fruits set per plant ranging from 46.7 to 65.3 and 36.5 to 58.5, respectively. In line with the previous results, flower and fruit set counts were the highest with the treatment of 1000 g of NPK compound Fertiliser per plant, with significant differences from other rates of applications and the control. Fruit set was also impressive with values between 81.3% and 89.6%. Interestingly, the fruit set ranged from 61.5% to 89.6% with NPK applications from 400 g to 1000 g per plant. But with higher applications (above 1000 g per plant), fruit set gradually declined. This trend is presumably a result of the negative impact of excessive NPK applications, promoting an increase in vegetation. Thus, the shoots at leaf axils turned into leaves, which limited the process of flowering and fruit setting for guava plants, as pointed out by Samant and Kishore (2020).

Guava Yield

The response of NPK compound Fertiliser, applied through irrigation system, on guava yield was studied by examining the key yield parameters - number of fruits set per plant, fruit drop (%), number of harvested fruits per plant, average fruit weight (g), and yield per plant (kg/plant), and per hectare (ton/ha) at harvest (Table 5). A quadratic effect of Fertiliser treatments was found, with an optimum in yield parameters at 1000 g/plant and a subsequent decrease, which was likely caused by unbalanced nutrition with a shift in plant physiology towards a higher vegetative growth and smaller yield in this particular guava variety. These findings are discussed in relation to the literature on guava and other fruit trees, highlighting the need for balanced fertilisation for yield optimisation.

The number of fruits per plant varied between 107.3 and 168.5 fruits per plant. This was observed as a quadratic response, where increasing Fertiliser rates from 400 to 1000 g/plant increased the fruit set, which peaked at 168.5 fruits per plant at 1000 g/plant. This was followed by a decline in fruit set as the rate increased further, producing 119.0 and

Table 5
Effect of NPK compound Fertiliser application through the drip irrigation system on guava yield

Fertiliser Rate (g/plant)	No. of Fruits/ Plant	Fruit drop (%)	Harvested Fruits/ plant	Average Fruit Weight (g)	Yield (kg/plant)	Yield (ton/ha)	Yield Increase vs. Control (%)
400	139.7 ^b	48.2 ^a	72.4 ^{bc}	300.8 ^e	21.8 ^{bc}	14.5 ^{bc}	27.6
600	147.6 ^b	47.4 ^a	77.6 ^b	315.3 ^{de}	24.5 ^b	16.3 ^b	35.6
800	147.1 ^b	45.3 ^a	80.5 ^b	321.5 ^{cd}	25.9 ^b	17.2 ^b	39.0
1000	168.5 ^a	45.5 ^a	91.8 ^a	368.4 ^a	33.8 ^a	22.5 ^a	53.3
1200	119.0 ^c	43.6 ^a	67.1 ^c	342.8 ^b	23.0 ^b	15.3 ^b	31.4
1400	119.2 ^c	43.8 ^a	67.0 ^c	336.5 ^{bc}	22.5 ^{bc}	15.0 ^b	30.0
Control	107.3 ^c	46.2 ^a	57.7 ^d	272.1 ^f	15.7 ^c	10.5 ^c	0.0
LSD _{0.05}	18.8	4.7	8.4	16.8	7.2	4.2	
CV %	8.3	6.5	7.3	8.9	6.3	5.9	

Note. Different letters within columns indicate significant differences at $P \leq 0.05$

119.2 fruits per plant with the 1200 and 1400 g/plant rates, respectively. This finding aligns with Widyastuti et al. (2023), who reported that higher rates of NPK from 250 to 500 g/plant increased the number of flowers and fruits of guava, but higher rates decreased fruit set, likely due to the promotion of vegetative growth that may inhibit fruit development.

There was significant variability in the number of fruits harvested and average fruit weight. The lowest were in the control treatment (57.7 fruits per plant, 272.1 g per fruit). With higher fertilisation (400-1000 g/plant), the number of fruits increased from 72.4 to 91.8 fruits per plant, and average fruit weight from 300.8 to 368.4 g per fruit, the latter being the maximum value. At higher levels (1200-1400 g/plant), both parameters decreased to 67.0 fruits per plant and 336.5 g per fruit at 1400 g/plant, which were not significantly different from 400 g/plant. This suggests that there is a threshold level of fertilisation at 1000 g/plant, above which excess nutrients may limit or affect the development of fruit set, which can be due to high nutrition stress or malformation of fruits due to hormone and/or growth-regulator disruption. Khan et al. (2018) also found the fruit set, fruit weight, and yield to be optimum at 828:240:288 g/tree of NPK Fertiliser. Increasing the amount of NPK Fertilisers beyond this threshold did not have notable differences in these parameters.

Trailing yield (fruit drop) was found to range from 43.6% to 48.2%, with no significant difference between treatments or control treatment (46.2%). There was a slight decrease in fruit drop as the Fertiliser amount increased from 400 g/plant (48.2%) to 1400 g/plant (43.8%), but the difference was not statistically significant. This indicates that factors other than Fertiliser rates, such as weather conditions (temperature and rainfall), are the main drivers of fruit drop.

Yield was determined as the average yield/plant and yield/hectare, from 15.7 to 33.8 kg/plant and 10.5 to 22.5 tons/ha. Every NPK application improved yield by 27.6-53.3%

over the control (using recommended practice for fertilisation). Maximum yield (33.8 kg/plant and 22.5 tons/ha) was observed with 1000 g/plant and was significantly ($P \leq 0.05$) higher than other levels of Fertilisers and treatment control. Over-fertilisation with higher rates (1200-1400 g/plant) led to a decline in yield (15.0-15.3 tons/ha), suggesting that over-fertilisation with high NPK rates can adversely affect guava yield. This is in agreement with Khan et al. (2018). Similarly, Nghi and Lan (2016) observed that treatment with 200 g of urea per plant produced optimum yield and sugar content; modest increases in urea rate (300 g/plant) did not result in significant increases in yield, irrespective of the total sugar content.

The quadratic response is consistent with other studies in guava and other fruit trees. Arshad (2015) found that NPK application at 1000 g/plant yielded maximum fruit numbers and weight, as well as yield of guava, in line with the optimal yield observed at 1000 g/plant in the current study. Moreover, Sharma et al. (2011) reported that supplementing nitrogen application with drip irrigation effectively increased guava yield relative to conventional fertilisation, suggesting that the use of irrigation to provide NPK is effective. These results are consistent with reports on other fruit trees, including mango, where a balanced NPK Fertiliser (1000 g N, 750 g P, and 750 g K per plant) maximised the yield and fruit quality, while higher rates decreased fruit retention because of excessive vegetative development (Azam et al., 2022).

Guava Fruit Quality

Nitrogen (N), phosphorus (P), and potassium (K) are vital components of guava plant development, affecting crop productivity and fruit quality. In this study, the application of NPK compound Fertilisers via drip irrigation was assessed in terms of the quality of guava fruit, as outlined in Table 6.

Table 6
Effect of NPK compound Fertiliser application through drip irrigation on guava fruit quality

Fertiliser Rate (g/plant)	Pulp Thickness (mm)	Core Thickness (mm)	Fruit Diameter (mm)	Fruit Flesh Weight Ratio (%)	TSS (%)
400	23.4 ^c	34.8 ^c	58.2 ^c	67.3 ^b	9.5 ^b
600	24.2 ^c	35.4 ^c	59.6 ^c	68.3 ^b	9.5 ^b
800	24.2 ^c	36.3 ^{bc}	60.6 ^c	66.6 ^b	9.6 ^{ab}
1000	29.1 ^a	38.1 ^b	67.2 ^b	76.6 ^a	9.8 ^a
1200	26.0 ^b	44.9 ^a	70.9 ^a	57.8 ^c	9.4 ^b
1400	24.9 ^{bc}	45.3 ^a	70.3 ^{ab}	55.0 ^c	9.5 ^b
Control	20.9 ^d	36.7 ^{bc}	57.6 ^c	57.2 ^c	9.1 ^c
LSD _{0.05}	1.7	2.2	3.2	3.8	0.2
CV %	3.8	3.2	3.1	3.3	1.1

Note. Different letters within columns indicate significant differences at $P \leq 0.05$

In the guava fruit, pulp thickness is a critical quality parameter, with increased pulp thickness leading to juicy fruit and high quality. With different levels of NPK Fertilisers, the pulp thickness varied from 20.9 to 29.1 mm. This measurement increased as the amount of Fertiliser increased, reaching a maximum level of 29.1 mm with the application of 1000 g/plant of NPK. But, there was a decrease in pulp thickness with NPK above 1000 g/plant. As for core thickness with seeds, the range was 34.8-45.3 mm. Maintaining an adequate level of NPK improved the nutrient uptake and translocation to the fruit and consequently increased the internode thickness and ratio of remaining fruit weight to flesh weight, and decreased pulp thickness. These results are consistent with Chavan et al. (2020), who found that the fruit pulp weight showed an increasing trend when applying nitrogen, phosphorus, and potassium (NPK) up to 300:150:150 g/plant but tended to decrease when applying higher NPK.

As for core thickness containing seeds, it varied from 34.8 to 45.3 mm. The maximum core thickness was recorded at the highest NPK levels (1400 g/plant), which were significantly higher than other formulations and the control treatment ($P \leq 0.05$). A linear increase was observed for core thickness with increasing rates of NPK Fertiliser, suggesting a positive association of NPK.

The fruit diameter increased with increasing Fertiliser rates and showed the highest thickness at 1200 g NPK/plant (70.9 mm) rates, then decreased at the highest Fertiliser rates. The ratio of fruit flesh weight varied from 57.2% to 76.6%, and it was highest at 1000 g NPK/plant, which was significantly higher than other fertilisation rates.

Increased TSS values indicate a higher content of sugar, which makes the fruit juice sweeter and tastier, giving an indication of increased fruit quality. Like other quality characters, the TSS value was significantly increased from 9.1% (control) to 9.5% by application of 400 g/plant. By increasing the Fertiliser doses to 1000 g/plant, the TSS value increased to the highest value (9.8%) and then began to decline with subsequent increases in Fertiliser doses. According to Kumar et al. (2009), increasing the amount of N and P did not improve the TSS of guava fruit. The TSS content, according to Arshad (2015), was found to be the highest (9.48%) with the level of fertilisation at 800:800:800 g NPK/plant and then decreased with the further addition of NPK.

Therefore, 1000 g/plant/year of NPK soluble compound Fertiliser with the drip irrigation system showed the highest pulp thickness, fruit flesh weight ratio, and TSS value, with significant differences in yield quantity as well as quality parameters from other levels of NPK.

CONCLUSION

This experiment demonstrated that using soluble NPK Fertiliser delivered via a drip irrigation system to guava trees increases their fruit set, yield and quality parameters. The

highest yield (22.5 tons/ha) and TSS (9.8 %) were found with an application rate of 1000 g/tree/year. The result should be applied to guava farmers in the ecological conditions of Nghe An Province.

But this finding was only in the first year and for one variety. More research needs to be done to test the economic viability of using soluble Fertilisers applied by drip irrigation in different guava varieties.

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REFERENCES

- Arshad, I. (2015). Integrated application of NPK fertilisers on the growth and yield of guava (*Psidium guajava* L.) in arid region of lower Sindh, Pakistan. *International Research Journal of Plant and Crop Sciences*, 2(1), 19-23. <https://doi.org/10.25046/aj010103>
- Azam, M., Qadri, R., Aslam, A., Khan, M. I., Khan, A. S., Anwar, R., Ghani, M. A., Ejaz, S., Hussain, Z., Iqbal, M. A., & Cheng, J. (2022). Effects of different combinations of N, P and K at different time interval on vegetative, reproductive, yield and quality traits of mango (*Mangifera indica* L.) cv. Dusehri. *Brazilian Journal of Biology*, 82, Article e235612. <https://doi.org/10.1590/1519-6984.235612>
- Challa, L. P., Behera, B. P., & Pradhan, P. C. (2021). Water requirement and nutrient management of guava (cv. Arka amulya) using drip under high density plantation in coastal Odisha. *The Pharma Innovation Journal*, 10(3S), 243-247. <https://doi.org/10.22271/tpi.2021.v10.i3Sd.6167>
- Chavan, M. T., Thutte, A. S., Kakade, A. R., & Solanke, A. A. (2020). Effect of levels of N, P, K on yield and quality of guava (*Psidium guajava* L.) under high density planting. *Journal of Pharmacognosy and Phytochemistry*, 9(6), 1290-1293.
- Fischer, G., & Melgarejo, L. M. (2021). Ecophysiological aspects of guava (*Psidium guajava* L.): A review. *Revista Colombiana de Ciencias Hortícolas*, 15(2), Article e12355. <https://doi.org/10.17584/rcch.2021v15i2.12355>
- Guleria, K., & Singh, D. (2023). Effects of levels of N:P: K on yield and quality of guava (*Psidium guajava* L.) under meadow orchard system. *International Journal of Plant & Soil Science*, 35(17), 467-472. <https://doi.org/10.9734/ijpss/2023/v35i173231>
- Gupta, P., Singh, D., Prasad, V. M., & Kumar, V. (2019). Effect of integrated nutrient management on growth and yield of guava (*Psidium guajava* L.) cv. Allahabad Safeda under high density planting. *Journal of Pharmacognosy and Phytochemistry*, 8(1), 1233-1236.
- Haokip, S. W., Shankar, K., & Lalrinnggheta, J. (2020). Climate change and its impact on fruit crops. *Journal of Pharmacognosy and Phytochemistry*, 9(1), 435-438.
- Janaki, D., Poorniammal, R., & Rajangam, J. (2020). Effect of drip fertigation on yield and quality of guava under ultra high density planting in Tamil Nadu. *International Journal of Chemical Studies*, 8(2), 2720-2722. <https://doi.org/10.22271/chemi.2020.v8.i2ap.9161>

- Khan, S., Kumar, A., & Sharma, J. R. (2018). Impact of NPK application on growth and yield of guava cv. Hisar Safeda. *International Journal of Current Microbiology and Applied Sciences*, 7(7), 286-290. <https://doi.org/10.20546/ijcmas.2018.707.034>
- Kumar, D., Pandey, V., Anjaneyulu, K., & Nath, V. (2009). Optimisation of major nutrient for guava yield and quality under east coastal condition. *Indian Journal of Horticulture*, 66(1), 18-21.
- Kumari, B., Prakash, S., Jaiswal, U. S., & Radheyshyam. (2018). Impact of nitrogen and boron on some vegetative, reproductive and quality of guava (*Psidium guajava* L.). *International Journal of Current Microbiology and Applied Sciences*, 7(4), 1752-1760. <https://doi.org/10.20546/ijcmas.2018.704.199>
- Nghi, Q. D., & Lan, V. T. Đ. (2016). Nghiên cứu một số biện pháp kỹ thuật nhằm rải vụ thu hoạch và nâng cao hiệu quả sản xuất ổi ODL1 trái vụ trên địa bàn thành phố Hà Nội [Research on technical measures for harvest extension and production efficiency of off-season ODL1 guava in Hanoi]. *Tạp chí Nông nghiệp và Phát triển nông thôn*, 1, 64-70.
- Rangare, N. R., Lal, N., Shiurkar, G., Banjare, K., Singh, P., Jangre, N., Sahu, N., & Patel, R. K. (2025). Impact of climate change on guava production: Challenges and adaptive strategies. *Applied Fruit Science*, 67(5), Article 404. <https://doi.org/10.1007/s10341-025-01637-8>
- Samant, D., & Kishore, K. (2020). Response of guava var. Arka Amulya to branch bending during winter and summer in the Eastern tropical region of India. *Journal of Environmental Biology*, 42(3), 720-726. <https://doi.org/10.22438/jeb/42/3/MRN-1581>
- Sharma, S., Patra, S. K., & Ray, R. (2011). Effect of drip fertigation on growth and yield of guava cv Khaja. *Environment and Ecology*, 29(1), 34-38.
- Shukla, A. K., Sarolia, D. K., Kumari, B., Kaushik, R. A., Mahawer, L. N., & Bairwa, H. L. (2009). Evaluation of substrate dynamics for integrated nutrient management under high density planting of guava (*Psidium guajava* L.) cv. Sardar. *Indian Journal of Horticulture*, 66(4), 461-464.
- Thirupathi, N., Kumar, M. R., Kumar, A. K., Sridhar, D., & Kumar, S. S. (2016). Studies on the effect of nitrogen, phosphorous and potassium on growth and yield of guava (*Psidium guajava* L.) cultivars under meadow system of planting. *Research in Environment and Life Sciences*, 9(2), 241-244.
- Widyastuti, R. A. D., Pujisiswanto, H., Warganegara, H. A., & Sutriana, E. S. (2023). Plant reproductive responses of guava ‘Crystal’ under different paclobutrazol and NPK fertiliser doses. *Journal of Tropical Crop Science*, 10(3), 238-243. <https://doi.org/10.29244/jtcs.10.03.238-243>

Dietary Evaluation of Ensiled *Leucaena leucocephala* Leaf Cultivars in Rabbit Feed Pellets on In Vitro Fermentation and Nutrient Digestibility

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ABSTRACT

This study evaluated *Leucaena leucocephala* (LL) cultivars, Tarramba (TRB) and Wandergraze (WDZ), as a partial replacement for alfalfa to reduce feed cost in the tropics. The study examined the effects of varying inclusion rates of LL cultivars in feed pellets on in vitro fermentation and nutrient degradability. Caecal fermentation was evaluated over 96 hours using caecal content from male Hycole rabbits. Six LL-based dietary treatments partially replacing alfalfa with 10%, 15%, and 20% of either TRB or WDZ, and a CON diet were evaluated, and the volume of gas released was recorded. Results showed that the CON diet significantly produced higher net gas volume (60.22 ml/200 mg DM), in vitro organic and dry matter digestibility (76.71% and 73.58%) and metabolizable energy (11.79 MJ/kg) than the LL-based diets ($p < 0.05$). Among the LL cultivars,

WDZ 10% yielded the highest net gas volume (49.57 ml/200 mg DM), in vitro organic and dry matter digestibility (67.68% and 64.97% and), and energy content (10.78 MJ/kg DM) while TRB-based diets produced the lowest net gas volume (33.12-38.38 ml/200 mg DM) and metabolizable energy (7.53-8.82 MJ/kg DM). The potential gas volume was highest in CON (76.49 ml) and lowest in TRB10% group (54.78 ml) ($p < 0.05$). In conclusion, WDZ 10% may represent an alternative forage resource for rabbits in high feed-cost regions, but performed

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less than an alfalfa-based diet in terms of fermentation and digestibility of nutrients. In vivo studies are recommended to examine the impacts of these cultivars on the production performance and overall health in rabbits.

Keywords: Anti-nutritional Factors, digestibility, hycole rabbit, in vitro fermentation, *Leucaena leucocephala*

INTRODUCTION

Rabbits are a prolific species of livestock in terms of meat production due to their fast growth response, superior feed conversion rate and efficient utilisation of forage diets (Ghosh et al., 2008). Nutritionally, rabbit meat is rich in protein, low in fat, high in omega-3 fatty acids and essential minerals including zinc, iron and calcium (Kumar et al., 2024). Hycole is a hybrid strain of rabbit known for its high production and reproductive traits, such as rapid weight gain, high birth rate, hybrid vigour, and adaptability to tropical environmental regions (Brahmantiyo et al., 2018). The production performance of rabbits depends essentially on nutrient composition and quality of diet, which critically influence voluntary feed consumption and digestibility (Getachew et al., 2005). Alfalfa, commonly used as a source of forage in conventional rabbit diets, is costly, prompting the need for a cheaper, sustainable alternative such as LL (Idris et al., 2023). *Leucaena leucocephala* is a leguminous forage high in crude protein (20 to 30%) and crude fibre (12 to 20%), with protein digestibility and amino acid structure comparable to alfalfa and soybean meal (Montoya-Flores et al., 2020; Nakamanee et al., 2019). The improved cultivars of LL have demonstrated strong potential as a dietary option for rabbits in tropical regions where alfalfa is expensive and cannot be cultivated in large quantities. Tarramba cultivar is high-yielding, persistent, drought-tolerant and psyllid resistant Dalzell (2006) whereas WDZ is characterised by high foliage production, superior establishment and high leaf-to-stem ratio (Dalzell, 2006). Comparing these varieties is critical to assess their impact on rabbit performance, feed utilisation efficiency and overall suitability as a cheap, sustainable tropical feed resource.

Leucaena inclusion in the rabbit diet has been reported to enhance substrate degradation, fermentation efficiency and microbial biomass production at a lower rate of inclusion (Debnath et al., 2016; Zain et al., 2019). At lower inclusion rates (10% to 15%), LL improved growth response, feed absorption efficiency and overall productivity (Adekojo et al., 2014). Processing methods like fermentation and thermal treatments have been found to reduce anti-nutritional compounds and improve fermentation efficiency (Utami & Akbar, 2025). Despite its nutritional advantages, its utilisation in monogastric animals' feed is restricted by the ANFs, primarily mimosine as well as condensed tannins (Fayemi et al., 2011). Mimosine is known to suppress microbial activity, alter fermentation dynamics, restrict protein metabolism and mineral utilisation (Mahanani et al., 2020). Tannins reduce

digestibility by attachment and forming complex units with dietary proteins (D'mello, 1992). High tannin levels can interfere with fermentation and reduce nutrient degradability (Zain et al., 2019). Additionally, tannins are known to limit protozoal activity and negatively influence fermentation (Albores-Moreno et al., 2019). Unprocessed LL leaves can impair feed conversion efficiency and nutrient utilisation due to reduced protein and dry matter digestibility (Nursiwi et al., 2018). Excessive intake of unprocessed LL leaves by rabbits is linked to restricted growth, liver damage, alopecia, and poor feed efficiency (Adedeji et al., 2013). A study by Adekojo et al. (2014) reported that processing methods like sun-drying, soaking, boiling, or fermentation can substantially reduce mimosine and tannin concentrations and improve the safety and nutritional value of LL leaf meal. Fayemi et al. (2011) also stated that treatments such as ensiling reduce tannin and mimosine content and improve nutrient utilisation and fermentation performance.

Despite the rich nutritional and forage yield of improved cultivars of LL used in this study, their potential as a major forage component in commercial rabbit feed formulation has not been evaluated. It is hypothesised that incorporating LL leaf cultivars in rabbit pellets will improve in vitro gas production, fermentation efficiency and degradability of nutrients compared to alfalfa-based diets. This will overcome the challenge of inadequate availability of feed and enhance sustainable, cost-effective rabbit production in the tropics. The findings of this study will contribute to the development of an alternative rabbit feed incorporating LL cultivars as a forage ingredient, suitable for commercial rabbit feed production. The study is designed to critically examine the impacts of varying inclusion levels of LL cultivars on gas production, substrate fermentation and nutrient degradability using the in vitro gas production protocol. The findings of this research will contribute to lowering the dependence on imported forage ingredients, reducing rabbit production cost and strengthening food security using locally grown feed resources in tropical regions.

MATERIALS AND METHODS

Preparation of *Leucaena leucocephala* Leaf Silage

The LL plants have been grown at Universiti Teknologi MARA (UiTM), Pahang, in Malaysia. Young leaves from both cultivars have been harvested after 60 days of growth. The leaves were then cut into 1 to 2 cm lengths to improve compaction efficiency and facilitate the fermentation process (Kung et al., 2018).

Prior to ensiling, additives have been added to improve silage quality and fermentation efficiency (De Pádua et al., 2014). Five percent molasses (fresh basis) was added as a source of readily fermentable carbohydrates to promote rapid pH reduction and lactic acid production. Urea was added at 1% to increase nitrogen availability and facilitate protein synthesis by microbes. *Lactobacillus plantarum*, an inoculant from lactic acid bacteria, was applied to improve lactic acid fermentation and depress undesirable microbial activity.

Additionally, a cellulase xylanase enzyme mixture (0.01% on a dry matter basis) was used to facilitate partial fibre degradation and improve substrate digestibility.

Following the addition of these components, the material was mixed thoroughly, tightly packed to exclude air in plastic silos, and stored under anaerobic conditions at temperatures ranging from 27 to 30 °C. During fermentation, the silage internal temperature was kept in the optimum range between 20 and 30 °C and regularly observed to enable efficient fermentation conditions (De Pádua et al., 2014).

The silage after six weeks was opened and dried in an oven at 60 °C for 48 hours to lower the moisture below 15%, thereby minimising microbial spoilage and nutrient loss. This ensiling process minimised the contents of mimosine and tannin to levels below 1%, as stated by Fayemi et al. (2011). The dried silage was subsequently crushed using a hammer mill with a 0.5mm sieve to ensure even particle size and improve pellet quality. The ingredients were pelleted to a uniform 3 mm diameter.

Formulations and Ingredient Content of Treatment Diets

The treatment diets in Table 1 were formulated and produced at the UiTM. Three inclusion levels of each cultivar contain 10%, 15%, and 20%, respectively. Only the alfalfa portion of the ingredient was partly replaced with LL silage. Other ingredients included rice bran, yellow corn, soybean meal, molasses, salt, vitamins, and minerals. All the ingredients used in the treatment diets were isonitrogenous and isocaloric, ensuring that only the forage composition varied. Alfalfa and LL serve as sources of fibre and protein. Yellow corn 10%, soybean meal 6% and rice bran 30% were maintained as consistent sources of protein and energy. Additives such as molasses 5%, calcium carbonate 2%, sodium chloride 0.5%, dicalcium phosphate 1.4%, and vitamin-mineral premixes were included uniformly to enhance palatability, mineral balance, and micronutrient supply. Synthetic amino acids like methionine 0.2%, threonine 0.2%, lysine 0.4% and arginine 0.4% were added to correct for limiting amino acids in the plant-based ingredients. These formulations demonstrated a rational and systematic approach to enhancing feed efficiency using locally available, nutrient-rich forages. By partially substituting alfalfa with LL cultivars, the diets will potentially reduce feed costs while maintaining adequate protein, energy, and mineral levels for optimal rabbit growth and health.

Proximate Analysis

Proximate analysis was conducted to determine the nutrient composition of treatment diets (Table 2). The samples of treatment pellets were oven-dried for 24 hours at 60 °C to reduce moisture content before performing proximate analysis. The samples were dried and ground using a 1 mm sieve to prepare them for nutrient analysis and the *in vitro* gas test. Dry matter (DM) analysis was conducted by drying samples in an oven overnight at 105 °C,

whereas ash content analysis was done by burning the dry feed samples in a 550 °C muffle furnace for a period of 4 hours. Crude fat (CF) analysis was done by the ether extraction (EE) protocol recommended by AOAC (2005). Nitrogen analysis was done according to the Kjeldahl procedure AOAC (2005), and the crude protein (CP) calculation followed using the formula $CP = N \times 6.25$. Organic matter (OM) was determined by deducting ash from dry matter. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) analyses were determined according to the method recommended by van Soest et al. (1991).

Table 1
Ingredient content of experimental treatments

Ingredients	Treatments					
	T1	T2	T3	T4	T5	T6
	TRB10%	TRB15%	TRB20%	WDZ 10%	WDZ 15%	WDZ 20%
Alfalfa (g/kg DM)	33	28	23	33	28	23
Leucaena (g/kg DM)	10	15	20	10	15	20
Soybean Meal (g/kg)	6	6	6	6	6	6
Yellow Corn (MJ/kg)	10	10	10	10	10	10
Rice Bran (MJ/kg)	30	30	30	30	30	30
Molasses (MJ/kg)	5	5	5	5	5	5
Calcium Carbonate (g/kg)	2	2	2	2	2	2
Dicalcium Phosphate (g/kg)	1.4	1.4	1.4	1.4	1.4	1.4
Sodium Chloride (g/kg)	0.5	0.5	0.5	0.5	0.5	0.5
Methionine (g/kg)	0.2	0.2	0.2	0.2	0.2	0.2
Lysine (g/kg)	0.4	0.4	0.4	0.4	0.4	0.4
Threonine (g/kg)	0.2	0.2	0.2	0.2	0.2	0.2
Arginine (g/kg)	0.4	0.4	0.4	0.4	0.4	0.4
Vitamin Premix (mg/kg)	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Premix (g/kg)	0.4	0.4	0.4	0.4	0.4	0.4
Total	100	100	100	100	100	100

Table 2
Nutrient composition of treatment diets

Nutrients (%)	Treatments					
	T1	T2	T3	T4	T5	T6
	TRB 10%	TRB 15%	TRB 20%	WDZ 10%	WDZ 15%	WDZ 20%
DM	91.32	90.45	91.71	91.87	91.48	92.03
CF	14.95	10.59	17.82	17.94	12.00	11.00
CP	17.01	15.40	16.47	17.70	12.85	16.57
EE	3.97	4.77	6.60	6.43	8.49	5.49
ADF	38.33	32.00	20.33	31.33	40.67	29.33
NDF	52.67	40.67	23.00	47.33	24.67	21.67

In Vitro Gas Production

Sampling of Caecal Content

Caecal samples were obtained from rabbits at GTG Agro Plantation Limited at Kajang in Malaysia. Slaughtering and sample collection were conducted during the early hours of the day at the facility. Prior to slaughter, the rabbits were deprived of feed but were offered water. Caecal samples were obtained from 6 healthy male rabbits approximately twelve weeks old, as described by Melillo (2007), with an average body weight of approximately 2.7kg. Obtaining samples from male rabbits of similar age and management condition ensures uniform caecal conditions, microbial activity, pH, and volatile fatty acid concentration, while preventing variations associated with female hormonal changes. Furthermore, while some studies use as few as 4 or as many as 10 animals, the use of 6 rabbits as a source of caecal samples provided statistically valid and reliable data while considering animal welfare and reducing animal use. The selected animals were euthanised humanely (Andreji et al., 2018), their digestive tract carefully removed, and the whole caecum excised. The caecal contents were immediately collected, mixed, stored in a vacuum flask under anaerobic conditions and transported to the Laboratory at the Faculty of Agriculture for subsequent in vitro gas assessment.

Preparation of Medium

Prior to the collection of caecal contents, all chemical solutions were prepared according to the methodology described by Menke & Steingass (1988), and stored in the laboratory until the day of the experiment. The solutions used included the following: Solution A (micro-mineral solution), consisting of 22.00g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 16.676g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.67g of $\text{CoCl}_3 \cdot 6\text{H}_2\text{O}$, and 13.33g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ diluted in 100 ml of distilled water (DW). Solution B (buffer solution) was prepared using 58.33g of NaHCO_3 and 6.67g of NH_4HCO_3 in 1000 mL of DW. Furthermore, solution C (macro-minerals) comprised 9.50g of Na_2HPO_4 , 10.33g of KH_2PO_4 , and 1.00g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ diluted in 1000 ml of DW.

Additionally, 0.17g of resazurin was diluted in 100 mL of DW to serve as an oxidation-reduction indicator. The reducing solution was made by diluting 6.67g of sodium hydroxide (1N NaOH) and 1.041g of $\text{Na}_2\text{S} \cdot 7\text{H}_2\text{O}$ in 100 ml of DW.

For experimental use, the medium was prepared based on the number of samples to be analysed. The composition of the medium included 378.82 ml DW, 0.10 ml Solution A, 189.41 ml Solution B, 189.41 ml Solution C, 39.96 ml reducing solution, and 0.27 ml sodium sulfide. The resulting mixture of solutions was flushed with CO_2 for 3 to 5 minutes to establish an anaerobic environment. Subsequently, 0.98 ml of resazurin was included, and the mixture was maintained under CO_2 until the mixture became colourless, indicating a reduced anaerobic state.

Preparation of Inoculum

The inoculum was prepared following the procedure formulated by Bovera et al. (2008). 200ml volume of caecal content was diluted with 200ml of medium and mixed for five minutes. The resulting mixture was then filtered via 6 layers of muslin cloth while continuously flushing with CO₂. The filtrate obtained was subsequently combined with an additional 200 ml of medium and homogenised for 20 seconds under continuous CO₂ flushing. The homogenised mixture was filtered again with 6 layers of muslin cloth. The filtrates from both filtration stages were pooled and kept at 39 °C under a CO₂ environment. The final inoculum preparation had a dilution ratio of 2:1 (medium:caecal material).

Determination of Gas Production

The gas production and measurement procedure was conducted in accordance with the protocol described by Menke and Steingass (1988). Briefly, about 200mg of the feed samples were placed in lubricated 100ml glass syringes. Each syringe was subsequently inoculated with 30ml of an anaerobic medium mixture with caecal inoculum. The syringes were incubated strictly under anaerobic conditions at 39 °C in a H₂O bath and continuously agitated for a total duration of 96 hours.

Gas volumes were recorded at times 0, 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 hours. Blank syringes were involved to account for gas released originating from the inoculum and medium, while standard concentrate samples were incorporated to monitor experimental consistency and deviations from normal expected values.

After the termination of incubation, samples were evaluated to determine pH, dry DM, ash, OM, OMD, and metabolizable energy (ME). The Net gas production, corrected using blank values and standardised at 24 hours of incubation, was calculated following the procedure outlined by Menke and Steingass (1988).

Determination of Caecal pH

After a 96-hour incubation period, caecal fluids were filtered through sintered glass. The pH of the filtered caecal content, retained in each glass syringe, was determined with a pH meter. Prior to use, the pH meter had been calibrated using standard solutions at pH 4, pH 7, and pH 10, respectively. Subsequently, the pH value of all the samples was recorded.

Determination of Nutrient Digestibility

Digestibility of In Vitro Dry Matter

The caecal fluids from glass syringes after 96 hours of water bath incubation were shifted into oven-dried, clean, previously weighed and labelled sintered glass filters. All the

glasses containing the fluids were vacuum filtered. Dry matter residues were calculated as a constant weight after drying at 105 °C.

The in vitro dry matter digestibility (IVDMD%) was determined according to the method of Tilley & Terry (1963) using Equation 1 as follows:

$$\text{IVDMD (\%)} = \left(\frac{\text{Initial DM} - \text{Residual DM}}{\text{Initial DM}} \right) \times 100 \quad [1]$$

Where:

Initial DM = sample weight before incubation

Residual DM = residual weight after incubation

Digestibility of In Vitro Organic Matter

The in vitro organic matter digestibility (IVOMD%) was determined following Equation 2, proposed by Menke et al. (1979).

$$\text{IVOMD (\%)} = 14.88 + 0.889\text{GP} + 0.45\text{CP} + 0.0651\text{XA} \quad [2]$$

Where:

GP = 24-hour net gas volume

CP = crude protein

XA = ash content

Evaluation of Metabolizable Energy

The metabolizable energy (ME) value of experimental feeds was estimated from gas volumes after a 24-hour incubation period. Gas volumes noted after 24 hours were standardised to 200 mg DM. The ME content was calculated from the predictive Equation 3 developed by Menke & Steingass (1988) for concentrate feed, expressed as:

$$\text{ME (MJ/kg DM)} = 1.06 + 0.157\text{GP} + 0.084\text{CP} + 0.22\text{CF} - 0.081\text{CA} \quad [3]$$

Where:

GP = net gas volume at 24 hours

CP = crude protein

CF = crude fat

CA = crude ash

This equation was derived from the Hohenheim gas test protocol, which has been validated through extensive research. It was established using data from 400 *in vivo* digestibility trials, combined with their corresponding *in vitro* gas production results (Menke & Steingass, 1988).

STATISTICAL ANALYSIS

The data were arranged in a Completely Randomized Design and subjected to one-way analysis of variance with R 4.5.2 statistical software tool. Variations between means were evaluated using the Least Significant Difference test. Statistical significance among treatment groups was considered at ($p < 0.05$).

RESULTS

In Vitro Gas Production of Treatments Diets at Various Incubation Times

The results in Table 3 on gas production trend indicated significant differences among treatments at different incubation times, reflecting variations in fermentability. Gas production increased progressively with incubation time across all diets, indicating continuous microbial activity. During the early incubation phase, WDZ-treated diets exhibited greater fermentation efficiency and produced gas levels comparable to those of the CON. In contrast, TRB-treated diets showed lower gas production, suggesting reduced microbial fermentation. The CON diet maintained the highest gas production throughout the study. Among the supplemented diets, WDZ treatments consistently outperformed TRB treatments at later incubation periods, indicating better substrate degradation and caecal microbial utilisation. Higher inclusion levels of WDZ produced similar fermentation responses, suggesting stable and efficient microbial activity. Overall, WDZ supplementation enhanced *in vitro* fermentation more effectively than TRB supplementation.

Net Gas Production, pH, In Vitro Digestibility and Metabolizable Energy of Treatment Diets

Treatments significantly affected net gas production, pH, ME and *in vitro* digestibility parameters Table 4. The CON diet consistently showed superior fermentation characteristics, energy availability, and digestibility compared to all treated diets, indicating better overall feed utilisation. Diets containing TRB generally recorded the poorest performance, with reduced gas production, lower pH, and decreased digestibility and metabolizable energy, suggesting less favourable caecal fermentation and nutrient utilisation. In contrast, diets containing WDZ performed better than the TRB-based diets, showing improved fermentation conditions, higher energy values, and better digestibility.

Table 3
In vitro gas production by treatment diets at different incubation times

Incubation Time (h)	Treatments						T7 CON	p-value
	T1	T2	T3	T4	T5	T6		
	TRB 10%	TRB 15%	TRB 20%	WDZ 10%	WDZ 15%	WDZ 20%		
T2	17.27 ^c ±1.21	16.87 ^c ±1.24	17.92 ^{bc} ±0.85	23.97 ^a ±1.61	21.80 ^{abc} ±2.55	22.42 ^{ab} ±1.98	20.50 ^{abc} ±2.89	*
T4	23.07 ^{bc} ±1.47	21.37 ^c ±1.41	23.17 ^{bc} ±0.96	28.27 ^a ±1.37	27.30 ^{ab} ±2.10	27.55 ^{ab} ±2.29	26.67 ^{abc} ±2.65	*
T6	27.70 ^{cd} ±1.88	27.20 ^d ±1.41	28.75 ^{bcd} ±0.75	35.00 ^a ±1.41	33.63 ^{ab} ±1.98	32.88 ^{abc} ±2.32	33.83 ^{ab} ±3.53	*
T8	30.93 ^{cd} ±2.09	30.53 ^d ±1.59	32.33 ^{bcd} ±0.82	38.23 ^a ±1.54	36.96 ^{ab} ±1.93	36.46 ^{abc} ±2.60	40.66 ^a ±2.96	**
T10	32.47 ^c ±2.29	32.37 ^c ±1.67	33.92 ^{bc} ±1.11	40.07 ^a ±1.44	39.07 ^{ab} ±1.91	38.42 ^{ab} ±2.50	43.67 ^a ±3.21	**
T12	34.20 ^{cd} ±2.08	34.00 ^d ±1.90	35.75 ^{cd} ±1.03	41.80 ^{ab} ±1.60	42.00 ^{ab} ±1.06	39.88 ^{bc} ±2.55	46.67 ^a ±3.48	**
T24	43.53 ^c ±2.67	44.13 ^c ±2.46	47.08 ^{bc} ±1.31	51.73 ^b ±1.33	51.21 ^b ±1.66	49.71 ^{bc} ±1.63	59.33 ^a ±4.04	**
T48	53.13 ^{cd} ±2.75	51.13 ^d ±2.99	52.58 ^{cd} ±3.82	61.53 ^b ±1.47	60.71 ^{bc} ±1.85	58.46 ^{bcd} ±1.56	71.16 ^a ±6.80	**
T72	58.87 ^{bcd} ±3.32	55.67 ^d ±3.76	56.92 ^{cd} ±5.48	67.67 ^{ab} ±1.55	66.55 ^{abc} ±1.82	63.80 ^{bcd} ±1.56	77.50 ^a ±7.37	**
T96	62.17 ^{bc} ±3.44	57.17 ^c ±4.67	59.67 ^{bc} ±6.72	71.07 ^{ab} ±1.51	70.30 ^{ab} ±1.78	67.05 ^{bc} ±1.68	80.84 ^a ±7.80	*

Note. Within each row, means assigned different superscript letters vary significantly ($p < 0.05$) using the LSD test, while those sharing similar letters are insignificantly different ($p > 0.05$). *h, hour; CON, Control; TRB; Tarramba; WDZ, Wandergraze; * $p < 0.05$ and ** $p < 0.01$

Table 4
Treatment effects on net gas production, pH, in vitro digestibility and metabolizable energy

Treatments	Parameters				
	Net GP (ml/200mg DM)	pH	ME (MJ/Kg DM)	Nutrient digestibility	
				IVDMD%	IVOMD%
T1 TRB 10%	33.12 ^d ±5.04	5.52 ^{bc} ±0.04	7.53 ^c ±0.79	51.84 ^c ±0.66	57.82 ^{cd} ±4.48
T2 TRB 15%	33.33 ^{cd} ±4.30	5.45 ^c ±0.03	7.7 ^c ±0.67	50.92 ^c ±0.58	57.20 ^d ±3.82
T3 TRB 20%	38.38 ^{bcd} ±2.22	5.55 ^b ±0.03	8.82 ^{bc} ±0.35	54.43 ^d ±0.57	62.30 ^{bcd} ±1.98

Table 4 (continued)

Treatments	Parameters				
	Net GP (ml/200mg DM)	pH	ME (MJ/Kg DM)	Nutrient digestibility	
				IVDMD%	IVOMD%
T4 WDZ	49.57 ^{ab}	5.69 ^a	10.78 ^a	64.97 ^b	72.68 ^{ab}
10%	±2.94	±0.01	±0.46	±0.54	±2.62
T5 WDZ	47.66 ^{ab}	5.58 ^b	10.51 ^{ab}	61.73 ^c	68.82 ^{bc}
15%	±3.64	±0.04	±0.57	±0.57	±3.23
T6 WDZ	45.54 ^{bc}	5.51 ^{bc}	9.81 ^{ab}	60.89 ^c	68.63 ^{bc}
20%	±4.46	±0.01	±0.70	±0.56	±3.97
T7 CON	60.22 ^a	5.72 ^a	11.79 ^a	73.58 ^a	81.71 ^a
	±7.39	±0.02	±1.16	±0.57	±6.09
<i>p</i> -value	**	***	**	***	**

Note. Figures with varying letters in a column imply differences significantly ($p<0.05$) with LSD; Values with similar letters in a column are not different significantly ($p>0.05$). GP, 24-hour net gas released; DM means Dry Matter; IVDMD denotes In Vitro Dry Matter Digestibility; ME, Metabolizable energy; IVOMD means In Vitro Organic Matter Digestibility; CON, Control; TRB, Tarramba; WDZ, Wandergraze; * means $p<0.05$; ** means $p<0.01$; *** means $p<0.001$.

Although their performance remained below that of the CON, the lower inclusion level of WDZ produced results that closely approached the CON treatment, indicating a comparatively better nutritional and fermentative potential.

In Vitro Fermentation Characteristics

The in vitro fermentation characteristics demonstrated in Table 5 indicated a significant difference among treatments. Gas production from rapidly soluble components was significantly higher in the CON diet, indicating better fermentation efficiency compared to the LL-based diets. In contrast, gas volume from the insoluble but fermentable fraction was clearly reduced in all experimental groups, indicating a lower extent of substrate degradation and microbial fermentation of structural or slowly digestible components compared with the CON diet. This reduction carried through to the overall gas production potential, where all modified diets consistently yielded lower total fermentative output, reflecting diminished overall substrate availability or fermentability. Despite these changes in fermentation extent, the fermentation rate remained stable across treatments, suggesting that the speed of microbial activity was not altered; rather, it was the extent of fermentation that was affected. Overall, the results point to a reduction in total fermentable substrate in experimental diets without affecting the kinetics of fermentation.

Table 5

In vitro fermentation characteristics and gas production dynamics

Parameters	Treatments							p-value
	T1 CON	T2 TRB 10%	T3 TRB 15%	T4 TRB 20%	T5 WDZ 10%	T6 WDZ 15%	T7 WDZ 20%	
a (ml)	8 . 4 8 ^a ±1.75	6 . 9 3 ^{a b} ±0.45	5 . 4 2 ^b ±0.75	5 . 5 6 ^b ±1.23	6 . 3 2 ^{a b} ±0.60	7 . 4 4 ^{a b} ±1.24	7 . 5 1 ^{a b} ±0.48	*
b (ml)	7 0 . 1 6 ^a ±6.70	5 1 . 2 9 ^b ±3.29	4 9 . 3 6 ^b ±3.15	5 0 . 9 8 ^b ±4.10	5 7 . 3 9 ^b ±1.55	5 7 . 6 1 ^b ±1.66	5 4 . 7 0 ^b ±0.65	**
c (ml/h)	0 . 0 7 ^a ±0.007	0 . 0 7 ^a ±0.006	0 . 0 8 ^a ±0.018	0 . 0 9 ^a ±0.022	0 . 0 8 ^a ±0.006	0 . 0 8 ^a ±0.005	0 . 0 9 ^a ±0.011	NS
a + b (ml)	7 6 . 4 9 ^a ±7.55	5 8 . 2 2 ^{b c} ±3.17	5 4 . 7 8 ^c ±3.84	5 6 . 5 4 ^{b c} ±5.33	6 5 . 8 7 ^{a b} ±1.44	6 5 . 0 5 ^{a b c} ±1.79	6 2 . 2 1 ^{b c} ±1.10	*

Note. Means within each row bearing dissimilar superscripts are significantly different using the LSD test ($p < 0.05$). In this context, a represents gas released from the rapidly soluble fraction, b denotes gas released from the insoluble fraction, and c indicates the constant rate of gas released from the insoluble component. The term a + b refers to the potentially degradable fraction. $p < 0.05$ denotes significant differences.

*CON, Control; TRB, Tarramba; WDZ, Wandergraze

DISCUSSION

This study assessed the potential of LL cultivars as a partial substitute for alfalfa in rabbit feed. The findings of this study will enhance the development of novel rabbit feed, including LL as a forage source, which can be applied in commercial rabbit feed production in the tropics, where conventional feed is costly and not adequately available. This study found that incorporating LL leaves into feed pellets for Hycole rabbits significantly influenced *in vitro* fermentation, as evidenced by the reduced gas production observed in LL-supplemented diets relative to the traditional alfalfa-based diet, which produced higher gas volume, indicating higher nutrient availability and fermentability. Gas production is mainly considered an indicator of microbial fermentative activity and substrate degradability. The lower gas volumes recorded in LL-based diets suggest reduced microbial degradation of nutrients and fermentability. This response may likely be associated with ANFs, mainly tannin, mimosine and other secondary metabolites in LL leaves, which can inhibit enzymatic activity and microbial attachment during fermentation (Petlum et al., 2019). Among the LL-based diets, the WDZ-treated diet produced higher gas volume and better digestibility than the TRB-based diet, indicating that diet composition significantly influenced microbial utilisation of nutrients. The higher fermentation recorded in WDZ diets may be attributed to lower structural fibre fractions and higher availability of fermentable carbohydrates, which could lower the inhibitory effects of tannins. In contrast, the lower gas volume in the TRB-based diets may reflect the combined effects of higher fibre and tannin content, which reduce nutrient degradation and microbial colonisation. Similar findings were reported

by Gaviria-Urbe et al. (2022), who found that inclusion of LL altered fermentation kinetics and decreased gas production based on diet composition and tannin content. Barros-Rodriguez et al. (2014), also reported that tannin-rich forages lower fermentation by reducing microbial degradation of carbohydrates and proteins.

Furthermore, reduced gas volume in LL diets may suggest lower methane-generating fermentation losses. Condensed tannins suppress methanogenic microorganisms and decrease hydrogen availability for methanogenesis, thereby diminishing methane production during fermentation (Petlum et al., 2019). A study by Tadesse et al. (2024) reported a linear drop in methane production with increasing inclusion of dried LL leaves in animal diets. Although LL inclusion reduced overall fermentability compared with the conventional diet, moderate inclusion in this study may provide environmental benefits via reduced methane emissions. The results further suggest that the response to LL inclusion is highly dependent on the diet composition, with WZ-based diets demonstrating a better balance between microbial activity, digestibility, potential methane mitigation and energy efficiency.

Tannins are secondary metabolites that significantly impair digestibility and nutrient utilisation in rabbits. Tannin reduces carbohydrate and protein digestibility by binding dietary proteins and inhibiting digestive enzymes, thus limiting microbial access to digestible nutrients, thereby depressing feed efficiency (Barros-Rodríguez et al., 2014). Tannins limit protozoal activity and negatively influence fermentation (Albores-Moreno et al., 2019). Tannin toxicity in rabbits offered high rates of LL demonstrates weight loss, hepatic congestion, poor feed conversion, alopecia and increased mortality. In rabbits, dietary inclusion of high tannin content LL above 1% reduced growth and overall productivity (Fayemi et al., 2011). The tannin content of the LL leaf, about 6.57% act as bactericides through attaching itself to bacterial membranes and reducing membrane permeability while depressing metabolic activity (Alduwish et al., 2025; Aydin, 2024; Khatoon et al., 2025).

Furthermore, the inclusion of LL in livestock feed raises significant health concerns, particularly in non-ruminant animals such as rabbits. Unlike ruminants, which harbour gut microorganisms including *Clostridium butyricum*, *Streptococcus lutetiensis*, and *Synergistes jonesii* capable of degrading mimosine and its toxic metabolites (3,4- and 2,3-dihydroxypyridine, DHP) into non-toxic compounds, rabbits lack such microbial detoxification systems (Derakhshani et al., 2016). Thus, consumption of mimosine-containing plants in rabbits can result in liver damage through oxidation, tyrosine metabolism disruption, infertility, impaired growth response and overall health. Mimosine is a non-protein amino acid present in leguminous forages, including LL, which can significantly influence protein synthesis (Mahanani et al., 2020). The cytotoxicity of mimosine occurs from its ability to interfere with the cell cycle, thereby inhibiting DNA

replication. It exhibits antibacterial properties via the attachment to bacterial proteins, interfering with the cell membrane integrity and hindering bacterial growth, causing bacterial cell death (Mohammed et al., 2015; Rosida et al., 2017). Additionally, it chelates vital metal ions such as iron and zinc, causing oxidative stress and ultimately inducing apoptosis. These toxic effects manifest as infertility, growth retardation, alopecia, liver damage and cataract formation.

Inclusion of LL at a moderate level may positively affect caecal fermentation by supplying fermentable proteins, but when incorporated in higher quantities, ANFs' effects reduce nutrient availability and enzyme action by microbes, hence inhibiting fermentation efficiency. Studies have shown that treatments such as heating and ensiling reduce tannin and mimosine content while improving nutrient utilisation for better fermentation performance (Fayemi et al., 2011). However, different studies have shown that lower inclusion rates of LL (10-15%) have been linked to better substrate degradation, while higher concentrations negatively affect fermentation efficiency and microbial biomass production (Debnath et al., 2016; Zain et al., 2019). A study by Al-Amin et al. (2019) and Debnath et al. (2016) highlighted that LL supplementation level at 10% enhanced fibre digestibility while lowering methane production, which benefits both animal productivity and environmental sustainability. These findings emphasised the importance of carefully adjusting LL levels in feed formulations to strike a balance between its nutritional benefits and the potential downsides of ANF content.

Furthermore, this study discovered a notable decrease in IVDMD and IVOMD percentage in LL-treated diets compared to the CON group. The reduction may be attributed to high fibre content and ANFs in the treated diets, which were known to depress microbial function and reduce the availability of dietary nutrients (Deaville et al., 2010; Patra & Saxena, 2011). This is consistent with the findings by Makkar (2003) and Min et al. (2003) who reported that higher tannin and fibre levels in LL can hinder microbial enzyme activity and reduce substrate accessibility. Diets with higher digestibility usually have less lignocellulosic fractions and a lower concentration of ANFs, which enhance microbial fermentation. Contrasting results were reported in dairy and ruminant studies, where 10-20% inclusion rates of LL maximised digestibility and feed efficiency and reduced the negative effects of ANFs (Permana et al., 2022). Low to moderate levels of LL inclusion (10-20%) have been shown to improve digestibility without harming microbial activity. For instance, 10% supplementation of LL leaf meals has been found to influence microbial degradation, boost digestibility, nutrient absorption and energy utilisation in rabbits and ruminants (Permana et al., 2022; Verdecia et al., 2020). However, high levels of inclusion, especially above 20%, have been associated with decreased digestibility and nutrient utilisation, mainly due to the negative effects of ANFs, which interfere with microbial degradation of carbohydrates and proteins (Nieves et al., 2004).

These findings emphasised the significance of optimising both the inclusion rate and variety of LL to improve feed efficiency. Among all the LL-supplemented diets tested, the Wdz variety at a 10% inclusion rate demonstrated the best results in terms of digestibility and energy output. The optimal performance of Wdz may likely stem from a well-balanced protein-to-ANFs ratio, which facilitates efficient microbial fermentation. Although LL may offer promising benefits for enhancing feed quality and fermentation, it must be used wisely. Higher inclusion rate, especially from the TRB variant, can impede nutrient bioavailability and limit nutrient absorption. Future research should focus on developing effective methods to lower ANF levels in TRB-based feed and to explore the long-term impacts of Wdz inclusion on rabbit health, growth, and reproduction. Finally, these findings may contribute to the significance of LL's role in sustainable feeding strategies and underscore its potential to improve nutrient utilisation while decreasing reliance on conventional feed ingredients. Furthermore, *in vivo* trials are recommended to validate these outcomes and refine inclusion rates to optimise LL's potential as a sustainable and efficient feedstuff for rabbits.

CONCLUSION

This study found that incorporating LL varieties in rabbit pellets significantly influenced *in vitro* fermentation, gas production, digestibility, and energy utilisation. The CON diet performed better than LL-supplemented treatments in cumulative net gas production, metabolizable energy production and *in vitro* digestibility. Among the experimental diets, Wdz-based diets perform better than TRB-based diets. The low performance of LL-based diets may be associated with ANFs and high fibre content. Overall, ensiled LL leaf cultivars, especially Wdz 10% inclusion rate, may represent a practical alternative forage resource for rabbits, particularly in regions affected by forage scarcity or high feed costs. However, under the conditions of this study, they perform less than the conventional alfalfa-based diet in terms of fermentation and digestibility of nutrients. Further *in vivo* studies are recommended to examine the impacts of Wdz and TRB cultivars on growth performance, nutrient digestibility, health, and productivity in Hycole rabbits.

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REFERENCES

- Adedeji, O. S., Amao, S. R., Ameen, Adedeji, T. A., & Ayandiran, T. A. (2013). Effects of varying levels of *Leucaena leucocephala* leaf meal diet on the growth performance of weaner rabbit. *Journal of Environmental Issues and Agriculture in Developing Countries*, 5(1), 5-9.
- Adekojo, Adama, T., & Ijaiya, A. (2014). Effects of different methods of processing *Leucaena leucocephala* leaf meal on growth performance and nutrient digestibility of rabbits. *International Journal of Agriculture and Forestry*, 4(5), 380-385.
- Ahmed, M. E., & Abdelati, K. A. (2009). Chemical composition and amino acids profile of *Leucaena leucocephala* seeds. *International Journal of Poultry Science*, 8(10), 966-970. <https://doi.org/10.3923/ijps.2009.966.970>
- Al-Amin, N., Astuti, A., & Widi, T. S. M. (2019). Nutrient intake and performance of male New Zealand White rabbits fed different level of *Leucaena* leaf meal in pelleted complete diets. *IOP Conference Series: Earth and Environmental Science*, 387(1), Article 012105. <https://doi.org/10.1088/1755-1315/387/1/012105>
- Albores-Moreno, S., Alayón-Gamboa, J. A., Miranda-Romero, L. A., Alarcón-Zúñiga, B., Jiménez-Ferrer, G., Ku-Vera, J. C., & Piñeiro-Vázquez, A. T. (2019). Effect of tree foliage supplementation of tropical grass diet on in vitro digestibility and fermentation, microbial biomass synthesis and enteric methane production in ruminants. *Tropical Animal Health and Production*, 51(4), 893-904. <https://doi.org/10.1007/s11250-018-1772-7>
- Alduwish, M. A., Alharthi, N. S., Alharbi, A. A., Albalawi, M., Obidan, A., Beyari, E. A., Al-Gheffari, H., Shakak, A. O., Allohibi, A., Almutairi, L. A., Almasoudi, S. H., Alsulami, R. N., Al-Doaiss, A. A., & Abu-Elsaoud, A. M. (2025). Evaluating the multifunctional therapeutic potential of phycocyanin: Antidiabetic, antioxidant, anticancer and antimicrobial effects on defense genes, metabolic, oxidative, and histopathological parameters in dithizone-induced diabetic rats. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 31(5), 619-634. <https://doi.org/10.9775/kvfd.2025.34347>
- Andreji, J., Fik, M., Arpášová, H., & Gunišová, S. (2018). Comparison of the growth performance of rabbits of the Hycole broiler hybrid in two breeding models. *Scientific Papers: Animal Science and Biotechnologies*, 51(2), 103-105.
- AOAC International. (2005). *Official methods of analysis of AOAC International* (18th ed.). Association of Official Analytical Chemists.
- Aydin, E. (2025). From deadly to life-saving effects: Antimicrobial and antibiofilm effects of *Tarantula cubensis* extract on bacterial and fungal pathogens. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 31(1), 59-67. <https://doi.org/10.9775/kvfd.2024.32854>
- Barros-Rodríguez, M. A., Solorio-Sánchez, F. J., Sandoval-Castro, C. A., Ahmed, A. M. M., Rojas-Herrera, R., Briceño-Poot, E. G., & Ku-Vera, J. C. (2014). Effect of intake of diets containing tannins and saponins on in vitro gas production and sheep performance. *Animal Production Science*, 54(9), 1486-1489. <https://doi.org/10.1071/AN14294>
- Barros-Rodríguez, M., Sandoval-Castro, C. A., Solorio-Sanchez, J., Sarmiento-Franco, L., Rojas-Herrera, R., & Klieve, A. V. (2014). *Leucaena leucocephala* in ruminant nutrition. *Tropical and Subtropical Agroecosystems*, 17(2), 173-183. <https://doi.org/10.56369/tsaes.2026>

- Bovera, F., Calabrò, S., Cutrignelli, M. I., Infascelli, F., Piccolo, G., Nizza, S., Tudisco, R., & Nizza, A. (2008). Prediction of rabbit caecal fermentation characteristics from faeces by in vitro gas production technique: Roughages. *Journal of Animal Physiology and Animal Nutrition*, 92(3), 260-271. <https://doi.org/10.1111/j.1439-0396.2007.00748.x>
- Brahmantiyo, B., Raharjo, Y. C., & Prasetyo, L. H. (2018). Production performance of HyCole, New Zealand White rabbits and its reciprocal. *Jurnal Ilmu Ternak dan Veteriner*, 22(1), 16-23. <https://doi.org/10.14334/jitv.v22i1.1590>
- Dalzell, S. A., Shelton, H. M., Mullen, B. F., Larsen, P. H., & McLaughlin, K. G. (2006). *Leucaena: A guide to establishment and management*. Meat and Livestock Australia.
- De Pádua, F. T., A. Fontes, C. A., C. Almeida, J. C., Deminici, B. B., Almeida Carlos, L. de, Neto, O. C., & de Oliveira, V. C. (2014). Fermentation characteristics of silage of sugar cane treated with calcium oxide, *Lactobacillus buchneri* and their associations. *American Journal of Plant Sciences*, 5(5), 636-646. <https://doi.org/10.4236/ajps.2014.55078>
- Deaville, E. R., Givens, D. I., & Mueller-Harvey, I. (2010). Chestnut and mimosa tannin silages: Effects in sheep differ for apparent digestibility, nitrogen utilisation and losses. *Animal Feed Science and Technology*, 157, 129-138. <https://doi.org/10.1016/j.anifeedsci.2010.02.007>
- Debnath, M., Khandakar, Z. H., Alam, M. A., & Heyamet, M. A. (2016). Effect of feeding different level of *Leucaena leucocephala* (ipil ipil) leaf meal for rabbit diet. *Asian-Australasian Journal of Bioscience and Biotechnology*, 1(2), 261-267. <https://doi.org/10.3329/ajbb.v1i2.61573>
- Derakhshani, H., Corley, S. W., & Al Jassim, R. (2016). Isolation and characterisation of mimosine, 3, 4 DHP and 2, 3 DHP degrading bacteria from a commercial rumen inoculum. *Journal of Basic Microbiology*, 56(5), 580-585. <https://doi.org/10.1002/jobm.201500590>
- D'Mello, J. P. F. (1992). Chemical constraints to the use of tropical legumes in animal nutrition. *Animal Feed Science and Technology*, 38, 237-261. [https://doi.org/10.1016/0377-8401\(92\)90105-F](https://doi.org/10.1016/0377-8401(92)90105-F)
- Fayemi, P. O., Friday, C. C., Onwuka, I., & Isah, O. A. (2011). Effects of mimosine and tannin toxicity on rabbits fed processed *Leucaena leucocephala* (Lam) De Wit. leaves. *African Journal of Agricultural Research*, 6(14), 3326-3332.
- Gaviria-Urbe, X., Castro-Montoya, J., Bolívar, D. M., Molina-Botero, I., Chirinda, N., Dickhoefer, U., Barahona, R., & Arango, J. (2022). The effects of two species of *Leucaena* on in vitro rumen fermentation, methane production and post-ruminal protein supply in diets based on *Urochloa* hybrid cv. Cayman. *Agronomy*, 12(3), Article 629. <https://doi.org/10.3390/agronomy12030629>
- Getachew, G., DePeters, E. J., Robinson, P. H., & Fadel, J. G. (2005). Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Animal Feed Science and Technology*, 123-124, 547-559. <https://doi.org/10.1016/j.anifeedsci.2005.04.034>
- Ghosh, S. K., Das, A., Bujarbaruah, K. M., Das, A., Dhiman, K. R., & Singh, N. P. (2008). Effect of breed and season on rabbit production under sub-tropical climate. *World Rabbit Science*, 16(1), 29 - 33. <https://doi.org/10.4995/wrs.2008.638>
- Idris, N. H., Muhammad, N. S., Abas, A., & Kasmin, M. N. (2023). Are Malaysians ready for rabbit meat? An exploratory to assess readiness of Malaysians to accept rabbit meat as an alternative protein source.

- Environment-Behaviour Proceedings Journal*, 8(SI15), 149-154. <https://doi.org/10.21834/e-bpj.v8iSI15.5099>
- Khatoon, A., Abidin, Z. U., Ali, A., Saleemi, M. K., Abbas, R. Z., Ijaz, M., Rehman, T. U., & Murtaza, M. (2025). Managing mycotoxins in animal/poultry feed through innovative control strategies: A review. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 31(5), 581-602. <https://doi.org/10.9775/kvfd.2025.34700>
- Kumar, P., Sharma, N., Narnoliya, L. K., Verma, A. K., Umaraw, P., Mehta, N., Ismail-Fitry, M. R., Kaka, U., Yong-Meng, G., Lee, S. J., & Sazili, A. Q. (2024). Improving quality and consumer acceptance of rabbit meat: Prospects and challenges. *Meat Science*, 219, Article 109660. <https://doi.org/10.1016/j.meatsci.2024.109660>
- Kung, L., Shaver, R. D., Grant, R. J., & Schmidt, R. J. (2018). Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *Journal of Dairy Science*, 101(5), 4020-4033. <https://doi.org/10.3168/jds.2017-13909>
- Mahanani, M. M. P., Kurniawati, A., Hanim, C., Anas, M. A., & Yusiati, L. M. (2020). Effect of (*Leucaena leucocephala*) leaves as tannin source on rumen microbial enzyme activities and in vitro gas production kinetics. *IOP Conference Series: Earth and Environmental Science*, 478(1), Article 012088. <https://doi.org/10.1088/1755-1315/478/1/012088>
- Makkar, H. P. S. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research*, 49(3), 241-256. [https://doi.org/10.1016/S0921-4488\(03\)00142-1](https://doi.org/10.1016/S0921-4488(03)00142-1)
- Melillo, A. (2007). Rabbit Clinical Pathology. *Journal of Exotic Pet Medicine*, 16(3), 135-145. <https://doi.org/10.1053/j.jepm.2007.06.002>
- Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D., & Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor in vitro. *The Journal of Agricultural Science*, 93(1), 217-222. <https://doi.org/10.1017/S0021859600086305>
- Menke, K. H., & Steingass, H. (1988). Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Animal Research and Development*, 28, 7-55.
- Min, B. R., Barry, T. N., Attwood, G. T., & McNabb, W. C. (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: A review. *Animal Feed Science and Technology*, 106, 3-19. [https://doi.org/10.1016/S0377-8401\(03\)00041-5](https://doi.org/10.1016/S0377-8401(03)00041-5)
- Mohammed, R. S., El Souda, S. S., Taie, H. A. A., Moharam, M. E., & Shaker, K. H. (2015). Antioxidant, antimicrobial activities of flavonoids glycoside from *Leucaena leucocephala* leaves. *Journal of Applied Pharmaceutical Science*, 5(6), 138-147. <https://doi.org/10.7324/JAPS.2015.50623>
- Montoya-Flores, M. D., Molina-Botero, I. C., Arango, J., Romano-Muñoz, J. L., Solorio-Sánchez, F. J., Aguilar-Pérez, C. F., & Ku-Vera, J. C. (2020). Effect of dried leaves of *Leucaena leucocephala* on rumen fermentation, rumen microbial population, and enteric methane production in crossbred heifers. *Animals*, 10(2), Article 300. <https://doi.org/10.3390/ani10020300>
- Nakamane, G., Harrison, S., Janthibordee, K., Srisomporn, W., & Phaikaew, C. (2019). Potential of *Leucaena* spp. as a feed resource for ruminant animals in Thailand. *Tropical Grasslands-Forrajes Tropicales*, 7(4), 449-454. [https://doi.org/10.17138/tgft\(7\)449-454](https://doi.org/10.17138/tgft(7)449-454)

- Nieves, D., Silva, B., Terán, O., González, C., & Ly, J. (2004). A note on the chemical composition and feeding characteristics of diets containing *Leucaena leucocephala* and *Arachis pinto*i for growing rabbits. *Livestock Research for Rural Development*, 16(12). <http://www.lrrd.org/lrrd16/12/niev16099.htm>
- Nursiwi, A., Ishartani, D., Sari, A. M., & Nisyah, K. (2018). Study on *Leucaena leucocephala* seed during fermentation: sensory characteristic and changes on anti-nutritional compounds and mimosine level. *IOP Conference Series: Earth and Environmental Science*, 102(1), Article 012093. <https://doi.org/10.1088/1755-1315/102/1/012093>
- Patra, A. K., & Saxena, J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *Journal of the Science of Food and Agriculture*, 91(1), 24-37. <https://doi.org/10.1002/jsfa.4152>
- Permana, I. G., Despal, Rosmalia, A., & Rahayu, M. D. (2022). Inclusion of different level *Leucaena* in dairy ration to balance rumen degradable and undegradable protein ratio. *IOP Conference Series: Earth and Environmental Science*, 1020(1), Article 012013. <https://doi.org/10.1088/1755-1315/1020/1/012013>
- Petlum, A., Paengkoum, P., Liang, J. B., Vasupen, K., & Paengkoum, S. (2019). Molecular weight of condensed tannins of some tropical feed-leaves and their effect on in vitro gas and methane production. *Animal Production Science*, 59(12), 2154-2160. <https://doi.org/10.1071/AN17749>
- Rosida, D. F., Djajati, S., Nilamayu, Z. A., & Rosida. (2017). Antibacterial activity of *Leucaena leucocephala* extracts on growth of *Escherichia coli*. *Advanced Science Letters*, 23(12), 12268-12271. <https://doi.org/10.1166/asl.2017.10618>
- Tilley, J. M. A., & Terry, R. A. (1963). A two-stage technique for the in vitro digestion of forage crops. *Grass and Forage Science*, 18(2), 104-111. <https://doi.org/10.1111/j.1365-2494.1963.tb00335.x>
- Utami, M. M. D., & Akbar, A. (2025). Enhancing nutrient intake, egg production, and egg quality by fermented *Leucaena leucocephala* leaf meal in a diet of laying quail. *Veterinary World*, 18(1), 133-140. <https://doi.org/10.14202/vetworld.2025.133-140>
- van Soest, P. J., Robertson, J. B., & Lewis, A. (1991). Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74(10), 3583-3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Verdecia, D. M., Herrera, R. S., & Ramírez, J. L. (2020). Effect of age of regrowth, chemical composition and secondary metabolites on the digestibility of *Leucaena leucocephala* in the Cauto Valley, Cuba. *Agroforest System*, 94(4), 1247-1253. <https://doi.org/10.1007/s10457-018-0339-y>
- Zain, M., Ningrat, R. W. S., Erpomen, Putri, E. M., & Makmur, M. (2019). The effects of leguminous supplementation on ammoniated rice straw based completed feed on nutrient digestibility on in vitro microbial protein synthesis. *IOP Conference Series: Earth and Environmental Science*, 287(1), Article 012018. <https://doi.org/10.1088/1755-1315/287/1/012018>

Effects of *Lactobacillus brevis* and *Lactobacillus plantarum* on Fermentation, Aerobic Stability, and Ruminal Digestibility of Sorghum Silage under Tropical Conditions

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ABSTRACT

This study aimed to evaluate the effects of *Lactobacillus brevis*, a heterofermentative bacterium, and *Lactobacillus plantarum*, a homofermentative bacterium, on reducing nutrient loss, extending aerobic stability, and improving the digestibility of sorghum silage under tropical conditions. Sorghum forage was harvested at 110 days and ensiled for 60 days with the following treatments: without inoculant (CON); *Lactobacillus brevis* FNCC0265 as a heterofermentative inoculant (LB); *Lactobacillus plantarum* FNCC0020 as a homofermentative inoculant (LP). Both LB and LP inoculants were applied at a concentration of 1×10^5 cfu/g of fresh forage. Each treatment used five mini silos (5 kg each) as replicates. The mini silo consisted of a plastic bag placed inside a 20-L container. After opening the silos, both LB and LP silages resulted in lower DM (dry matter) loss than the CON silage ($p < 0.05$). The concentration of acid detergent fibre, organic matter, crude

protein and neutral detergent fibre was not impacted by inoculant application. According to the fermentation characteristics, both LB and LP silages showed a lower pH, along with increased lactate and LAB (lactic acid bacteria) count compared to CON silage ($p < 0.05$). In addition, mould was not detected in LB and LP silages. Acetate concentration and aerobic stability were higher in LB silage than in LP and CON silages. In the rumen, LP and LB silage showed greater *in vitro* organic matter digestibility than CON silage ($p < 0.05$). This study concluded that both *Lactobacillus brevis* and *Lactobacillus*

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plantarum improved silage quality and *in vitro* ruminal digestibility, while *Lactobacillus brevis* additionally enhanced aerobic stability of sorghum silage under tropical conditions.

Keywords: Aerobic stability, *in vitro* digestibility, *Lactobacillus brevis*, *Lactobacillus plantarum*, organic acid, sorghum silage, tropical climate

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) has emerged as a promising alternative forage crop for ruminant production due to its adaptability to drought-prone environments, high biomass yield, and competitive nutritive value compared with conventional forages such as corn (Reddy & Blümmel, 2020). Sorghum can replace corn as a forage crop for dairy and beef cattle in tropical regions, and its heat tolerance enables sorghum to thrive in such environments (Singh et al., 2014). Sorghum is widely recognised as a potential feed resource in tropical areas facing seasonal forage shortages (Dahlya et al., 2025; Pazla et al., 2025). However, its use in ruminants is limited by the presence of antinutritional factors. Antinutritional aspects in sorghum, including tannins and cyanogenic glycosides (HCN), reduce nutrient use and palatability in animals (Etuk et al., 2012). Previous studies have reported that ensiling can reduce tannin and HCN levels while preserving nutrients (Ardiansyah et al., 2025; Etuk et al., 2012; Fitriani et al., 2024). Thus, ensiling sorghum is an effective strategy to enhance its utilisation in ruminant feeding systems.

Forage is abundant during the rainy season but becomes scarce and declines in quality during the dry season (Cooke et al., 2025). Silage technology helps stabilise supply, reduce anti-nutritional factors in sorghum, and preserve roughage quality (Fitriani et al., 2024; Selim et al., 2024). However, tropical silage production is hindered by humidity, forage moisture, and high temperature (Ardiansyah et al., 2025; Bernardes et al., 2018). These conditions cause silages to undergo rapid spoilage and aerobic deterioration, which decreases their nutritional value (Bernardes et al., 2018; Borreani et al., 2018; Kung et al., 2018).

LAB (lactic acid bacteria) inoculants are widely utilised to enhance the quality of nasilage in tropical climates (Bernardes et al., 2018; Fitriani et al., 2024). Without them, heat and moisture can promote butyrate production by *Clostridium* (Bernardes et al., 2018; Kung et al., 2018; Muck et al., 2018; Paradhipta et al., 2019). LAB inoculants modify the fermentation characteristics of silage during ensiling. Homofermentative LAB, such as *Lactobacillus plantarum*, primarily ferments soluble carbohydrates into lactic acid, which results in decreasing pH and suppressing harmful microbes (Arriola et al., 2011; Queiroz et al., 2013). Heterofermentative LAB, like *Lactobacillus brevis*, produce acetic acid, which has antifungal properties that improve the silage aerobic stability during the feed-out phase

(Holzer et al., 2003; Paradhipta et al., 2021). Therefore, the current study was designed to evaluate the effects of hetero and homofermentative LAB as inoculants on nutrient loss, aerobic stability, fermentation characteristics, and ruminal digestibility of sorghum silage under tropical situations.

MATERIAL AND METHODS

Silage Production

Sorghum forage (*Sorghum bicolor* L. Moench) with variety Samurai 2 was planted at Bantul Region, Province of Daerah Istimewa Yogyakarta (-7.866458, 110.408891). A total of 100 kg of sorghum forage was harvested at day 110, containing 27.7% of dry matter (DM). Forage was chopped to a length of 3-5 centimetres and ensiled in 20-litre mini silos (5 kg) following a previously published method (Paradhipta et al., 2024). A plastic bag placed inside a container was used as a mini silo with 60 cm of diameter. The fermentation was conducted for 60 days with different inoculant treatments. The treatments consisted of without inoculant (CON); *Lactobacillus brevis* FNCC0265, as a heterofermentative employed at 1×10^5 cfu/g of forage (LB); *Lactobacillus plantarum* FNCC0020 (LP), as a homofermentative employed at 1×10^5 cfu/g of forage. Each treatment used five mini silos as replicates. All silos were placed at the feed warehouse under semi-field conditions with temperatures approximately 27.4°C to 33.8°C. Both *L. brevis* and *L. plantarum* were obtained from the Centre for Food and Nutrition Studies of Universitas Gadjah Mada. After the ensiling period for 60 days, 200 g of each sample was taken for *in vitro* ruminal digestibility tests and chemical composition (Ardiansyah et al., 2025; Fitriani et al., 2024; Paradhipta et al., 2024). For fermentation characteristics, approximately 20 g of silage was extracted, while aerobic stability was evaluated using 1 kg samples incubated under aerobic conditions in 20 L mini silos (Ardiansyah et al., 2025; Fitriani et al., 2024; Paradhipta et al., 2019).

Chemical Compositions

Samples of fresh sorghum and silage were dried at 55°C for 48 hours and then ground using a Willey mill to pass through a 1 mm sieve. These were analysed for chemical composition and *in vitro* digestibility. DM (dry matter) content was measured after drying at 105°C for 24 h (Association of Official Analytical Chemists [AOAC], 2005; method 934.01). Organic matter (OM) was quantified by ashing at 600°C for 2 hours (AOAC, 2005; method 942.05). CP (crude protein) was measured using the Kjeldahl method (AOAC, 2005; method 954.01), while EE (ether extract) was measured using the Soxhlet method (AOAC, 2005; method 920.39). Fibre fractions, including NDF (neutral detergent fibre) and ADF (acid detergent fibre), were analysed with a fibre analyser (Ankom A200, USA) based on the AOAC method (AOAC, 2005; method 973.18).

Fermentation Characteristics

Silage fermentation parameters were determined by blending 180 g of the sample with 200 mL of distilled water for 30 s, followed by filtration through double-layered gauze. The extract was used for ammonia, pH, VFA (volatile fatty acid), and lactate measurements. A pH-meter was utilised to analyse the value of pH (Ohaus AB23PH-F, China). Ammonia concentration was assessed colourimetrically according to a previous study (Chaney & Marbach, 1962).

Volatile fatty acid and lactate concentrations were analysed with HPLC (high-performance liquid chromatography). Prior to HPLC analysis, aqueous samples were centrifuged at $12,000 \times g$ for 15 min to remove suspended solids and proteins. The supernatant was diluted with Milli-Q water, recentrifuged, and filtered through a $0.22 \mu\text{m}$ syringe filter into HPLC vials. The mobile phase comprised an acidified phosphate buffer (pH 2.0, adjusted with orthophosphoric acid) and a mixture of acetonitrile and methanol. The acidic buffer minimised fatty acid ionisation, thereby improving retention and separation on a reversed-phase C18 column. The method allowed accurate determination of the target fatty acids. The analysis was performed using a diode array detector (LC-2030C, Shimadzu, Japan) and a Shim-pack GIST C18 column, following methods outlined by Vargas et al. (2020) and Reuter et al. (2015). The column temperature was maintained at 30°C , the injection volume was applied at $20 \mu\text{L}$, and the working wavelength was 210 nm with a flow rate of 0.750 mL/min .

Microbial Count

Silage extracts were serially diluted up to 10^{-7} , and appropriate dilutions (10^{-5} to 10^{-7}) were used to enumerate LAB, yeast, and mould populations. LAB were cultured on MRS agar, and yeast and mould on PDA, with all assays conducted in triplicate. Plates of MRS were incubated using low oxygen in a CO_2 incubator at 30°C for 48 hours, whereas plates of PDA were incubated aerobically at 37°C for 72 hours. The difference between yeast and mould was based on morphological characteristics such as appearance and filamentous growth. Microbial populations were expressed as $\log_{10} \text{ cfu/g}$ (Paradhipta et al., 2021).

Aerobic Stability

Aerobic stability was evaluated by exposing 1 kilogram of silage to aerobic conditions in 20 L mini silos. Temperatures of both the ambient environment and the silage were measured every hour. Aerobic deterioration was considered to occur when the silage temperature increased by more than 2°C above the ambient temperature (Paradhipta et al., 2021).

***In vitro* Ruminal Digestibility**

Rumen fluid was collected from two fistulated Balinese cattle with an average body weight of 300 kg. During maintenance, all animals were fed with the standard diet to ensure an isonitrogenous and isoenergetic condition. The standard diet consisted of a 3:7 concentrate-to-forage diet (DM basis), containing 12% of CP and 10 kcal/kg of metabolizable energy. All animal care procedures and *in vitro* analyses were approved by the Ethics Committee of LPPT, Universitas Gadjah Mada (No. 00007/III/UN1/LPPT/EC/2024). Approximately 500 mL of rumen fluid was obtained before feeding in the morning, placed into anaerobic bottles, and transported to the laboratory for analysis. The buffer for the rumen was created by combining rumen fluid and McDougall solution at a ratio of 1:4. Approximately 0.5 g of dried silage sample was incubated with 40 millilitres of rumen buffer in CO₂-flushed bottles, which were then sealed and maintained at 39°C for 48 hours, with agitation performed every 8 hours. All of the procedures followed the method of Tilley & Terry (1963). Each treatment was applied in triplicate, including two blanks and two standard samples (Pangola grass). After incubation, the contents of the incubation bottle were filtered through Gooch crucibles, and the residues were used to determine *in vitro* DM digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD). The rumen buffer pH was determined using a pH meter (Ohaus AB23PH-F, China).

The results were expressed as IVDMD and IVOMD. The rumen buffer after incubation was collected to measure the pH value using a pH meter (Ohaus AB23PH-F, China).

Statistical Analysis

A completely randomised design (CRD) with the PROC GLM (General Linear Model) procedure in SAS version 9.4 was used to analyse data. The model of statistics was $Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ij} represents the observed response, μ denotes the overall mean, T_i the treatment impact of inoculant, and e_{ij} the random error. Each silo was considered the experimental unit ($n = 5$ per treatment). Analytical measurements, including fermentation characteristics, microbial counts, and *in vitro* ruminal digestibility, were conducted in triplicate, and the resulting values were averaged within each silo prior to statistical analysis. Post hoc comparisons were carried out using Tukey's test, with significance declared at $p \leq 0.05$.

RESULTS

Across all treatments, sorghum forage in the current analysis contained 27.7% DM, 90.3% OM, 7.74% CP, 1.45% EE, 48.3% NDF, and 27.8% ADF (Table 1). After ensiling for 60 days, the application of inoculant affected DM loss after ensiling (Figure 1). Both LB and LP silages had lower DM loss than CON silage ($p = 0.005$; 1.17% and 1.27% vs. 1.81%).

In addition, both LB and LP silages also had higher concentrations of DM after ensiling ($p = 0.049$; 27.0% and 27.2% vs. 26.3%) (Table 2). The concentration of EE was greater in LB silage than CON ($p = 0.036$; 3.11% vs. 2.01%), while LP silage did not differ compared to others. The concentration of OM, CP, NDF, and ADF from sorghum silage was not impacted by the application of inoculant. The means of OM, CP, NDF, and ADF from all treatments were 90.5%, 6.27%, 47.4%, and 37.6%, respectively.

Applications of LB and LP resulted in lower silage pH ($p = 0.045$; 3.58 and 3.57 vs. 3.69) than application of CON (Table 3). Ammonia was not impacted by the application of the inoculant. The mean of ammonia-N in all silages was 0.03%. The highest lactate concentration was reported in LP silage, followed by LB silage, and the lowest was in CON silage ($p = 0.002$; 3.03% vs. 2.68% vs. 2.26%). The concentration of acetate was greater in LB silage compared to in LP and CON silage ($p = 0.047$; 3.46% vs. 3.05% and 3.07%). Butyrate was not detected in all silages.

Table 1
Chemical compositions of sorghum forage before ensiling (% DM)

Item	
Dry matter	27.7 ± 0.32
Organic matter	90.3 ± 0.51
Crude protein	7.74 ± 0.12
Ether extract	1.45 ± 0.02
Neutral detergent fibre	48.3 ± 0.23
Acid detergent fibre	37.8 ± 0.19

Note. DM = dry matter

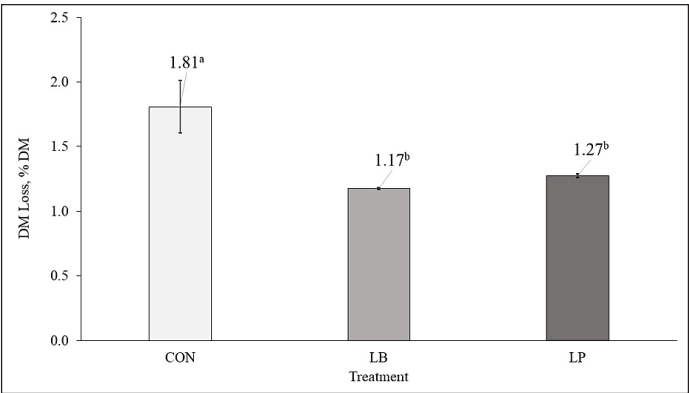


Figure 1. Effects of hetero and homofermentative inoculants on dry matter loss of sorghum silage ensiled for 60 days

Note. CON = silage without inoculant; LB= silage with *Lactobacillus brevis* FNCC0265 at 1×10^5 cfu/g of forage; LP = silage with *Lactobacillus plantarum* FNCC0020 at 1×10^5 cfu/g of forage; ^{a,b}Means with different superscript letters are significantly different ($p < 0.05$)

Table 2
Effects of hetero and homofermentative inoculants on chemical compositions of sorghum silage ensiled for 60 days (% DM)

Item	Treatment ¹			SEM	p-value
	CON	LB	LP		
Dry matter	26.3 ^b	27.0 ^a	27.2 ^a	0.518	0.049
Organic matter	90.2	91.0	90.4	0.733	0.212
Crude protein	6.24	6.29	6.30	0.483	0.985
Ether extract	2.01 ^b	3.11 ^a	2.64 ^{ab}	0.584	0.036
Neutral detergent fibre	48.0	47.1	47.2	3.253	0.920
Acid detergent fibre	38.7	37.6	36.4	2.980	0.511

Note. ¹CON = silage without inoculant; LB = silage with *Lactobacillus brevis* FNCC0265 at 1 × 10⁵ cfu/g of forage; LP = silage with *Lactobacillus plantarum* FNCC0020 at 1 × 10⁵ cfu/g of forage
*DM = dry matter; SEM = standard error of mean; ^{a,b} Means with different superscript letters in a row are significantly different (p < 0.05)

Table 3
Effects of hetero and homofermentative inoculants on fermentation characteristics of sorghum silage ensiled for 60 days

Item	Treatment ¹			SEM	p-value
	CON	LB	LP		
pH	3.69 ^a	3.58 ^b	3.57 ^b	0.007	0.045
Ammonia-N, % DM	0.03	0.03	0.04	0.008	0.355
Lactate, % DM	2.26 ^c	2.68 ^b	3.03 ^a	0.118	0.002
Acetate, % DM	3.05 ^b	3.46 ^a	3.07 ^b	0.136	0.047
Propionate, % DM	ND	ND	ND	NA	NA
Butyrate, % DM	ND	ND	ND	NA	NA

Note. ¹CON = silage without inoculant; LB = silage with *Lactobacillus brevis* FNCC0265 at 1 × 10⁵ cfu/g of forage; LP = silage with *Lactobacillus plantarum* FNCC0020 at 1 × 10⁵ cfu/g of forage
*DM = dry matter; SEM = standard error of mean; ND = not detected; NA = not applicable; ^{a,b} Means with different superscript letters in a row are significantly different (p < 0.05)

In microbial characteristics, LB and LP silages had greater LAB count (p = 0.003; 7.42 log₁₀ cfu/g and 7.45 vs 7.23 log₁₀ cfu/g), but lower mould count (p = 0.003; ND and ND vs 3.08 log₁₀ cfu/g) than CON silage (Table 4). Mould was not detected in LB and LP silages. The yeast count was not impacted by the application of inoculant. The mean count of yeast from all silages was 5.24 log₁₀ cfu/g. After the silo was opened, LB silage had a higher aerobic stability than CON and LP silages (Figure 2).

In the rumen, application of inoculant did not affect ruminal pH and IVDMD (Table 5). The means of rumen pH and DMD from all silages were 6.52 and 54.2%, respectively. However, LB and LP silages had a greater IVOMD compared to CON silage (p = 0.007; 64.7% and 63.8% vs. 61.8%).

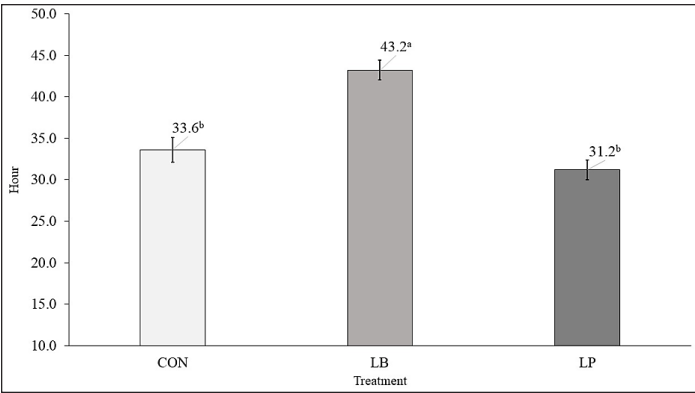


Figure 2. Effects of hetero and homofermentative inoculants on aerobic stability of sorghum silage ensiled for 60 days

Note. CON = silage without inoculant; LB= silage with *Lactobacillus brevis* FNCC0265 at 1×10^5 cfu/g of forage; LP = silage with *Lactobacillus plantarum* FNCC0020 at 1×10^5 cfu/g of forage; a,bMeans with different superscript letters are significantly different ($p < 0.05$)

Table 4
Effects of hetero and homofermentative inoculants on microbial counts of sorghum silage ensiled for 60 days ($\log_{10}$ cfu/g)

Item	Treatment ¹			SEM	p-value
	CON	LB	LP		
Lactic acid bacteria	7.23 ^b	7.42 ^a	7.45 ^a	0.070	0.003
Yeast	5.30	5.20	5.22	0.010	0.506
Mold	3.08 ^a	ND ^b	ND ^b	1,073	0.002

Note. ¹CON = silage without inoculant; LB= silage with *Lactobacillus brevis* FNCC0265 at 1×10^5 cfu/g of forage; LP = silage with *Lactobacillus plantarum* FNCC0020 at 1×10^5 cfu/g of forage

*DM = dry matter; SEM = standard error of mean; ND = not detected; NA = not applicable; ^{a,b} Means with different superscript letters in a row are significantly different ($p < 0.05$)

Table 5
Effects of hetero and homofermentative inoculants on ruminal digestibility of sorghum silage ensiled for 60 days

Item	Treatment ¹			SEM	p-value
	CON	LB	LP		
Rumen pH	6.47	6.54	6.55	0.970	0.416
IVDMD, % of DM	53.2	54.4	55.0	2.125	0.567
IVOMD, % of DM	61.8 ^b	64.7 ^a	63.8 ^a	0.660	0.007

Note. ¹CON = silage without inoculant; LB= silage with *Lactobacillus brevis* FNCC0265 at 1×10^5 cfu/g of forage; LP = silage with *Lactobacillus plantarum* FNCC0020 at 1×10^5 cfu/g of forage

*IVDMD = *in vitro* dry matter digestibility; IVOMD = *in vitro* organic matter digestibility; DM = dry matter; SEM = standard error of mean; ^{a,b} Means with different superscript letters in a row are significantly different ($p < 0.05$)

DISCUSSION

The chemical compositions of sorghum forage harvested at 110 days in the current analysis were within the normal range, consistent with other studies (Ardiansyah et al., 2025; Fitriani et al., 2024). After ensiling, the use of microbial inoculant in this study improved nutritional quality. According to findings, it was shown that the application of both homo and hetero LAB could decrease the DM loss of sorghum silage. This result was in line with several previous studies (Chotimah et al., 2024; Lee et al., 2021; Paradhipta et al., 2019, 2020). In addition, the level of EE was greater in LB silage than in CON silage, which indicated less lipolysis during ensiling due to the low pH condition (Table 3). The higher acetate concentration and lower pH observed in LB silage (Table 3) might have suppressed undesirable microbial activity, including lipolytic microbes and reduced nutrient degradation during fermentation (Liu et al., 2018; Kung et al., 2018; Lee et al., 2021). Furthermore, the lower DM loss observed in LB silage could have contributed to the relatively greater EE concentration after ensiling. In general, the decrease in nutrient loss by inoculant application was supported by the result of organic acid production (Table 3). Acetate generated by LB had a role as an antimicrobial agent (Holzer et al., 2003), while lactate generated by LP could establish an acidic condition that is unfavourable for the growth of unwanted microbes (Ardiansyah et al., 2025; Lee et al., 2019). The application of LB and LP had no effects on fibre fractions such as NDF and ADF because neither inoculant exhibited fibrolytic activity.

In fermentation characteristics, all inoculated silages resulted in lower pH than the CON silage. This result of pH was caused by higher production of organic acids (lactate and acetate) in LB and LP silages (Table 3). According to previous studies, the application of homofermentative generally increases lactate concentration, while the application of heterofermentative can improve acetate concentration (Arriola et al., 2011; Fitriani et al., 2024; Paradhipta et al., 2019). This is consistent with the results of lactate and acetate in this current study. The level of ammonia-N was not affected in the present study. This occurred because the pH was acidic enough to inhibit proteolytic activity (pH less than 4) (Tao et al., 2012). Moreover, the concentration of CP after ensiling did not differ among silages, which corroborated the findings of this study.

In this examination, the production of acetate was higher than the production of lactate in all silages. Generally, lactate is considered the main fermentation product. However, several previous studies also reported that a higher concentration of acetate than lactate in sorghum silage production conducted in tropical conditions (Ardiansyah et al., 2025; Fitriani et al., 2024). Although *Lactobacillus plantarum* is classified as a homofermentative LAB, the relatively high acetate concentration observed in LP silage suggests that fermentation may not have been exclusively dominated by the inoculated strain. Epiphytic heterofermentative LAB, enterobacteria, or yeasts naturally present on the forage may also

have contributed to acetate production during ensiling (Li et al., 2019; Muck et al., 2018). In addition, the inoculation level used in this study (1×10^5 cfu/g forage) might not have been sufficient to completely outcompete the native microflora under tropical conditions (Arriola et al., 2011; Kleinschmit et al., 2005).

Mainly, forage in tropical regions contains high moisture content, which could affect the ensiling process and organic acid production during incubation. Moreover, the temperature in a tropical climate was high, approximately 30°C - 33°C, during the incubation of silage. Similar to the present study, ensiling high-moisture forage at high temperature was reported to increase the potential of acetate production (Kim & Adesogan, 2006; Li et al., 2019). The microbial community has a pivotal role in acetic acid production. High-moisture silages favour *Lactobacillus* and *Enterobacter*, which can increase acetic acid production. In addition, *Clostridium* can produce butyric acid, thereby disrupting lactic fermentation (Li et al., 2019; Kung et al., 2018; Paradhipta et al., 2019). Despite high moisture, butyrate was not found in the silages. Their pH remained less than 4, sufficiently acidic to inhibit *Clostridium* growth (Borreani et al., 2018; Kung et al., 2018; Ogunade et al., 2018). However, parameters such as water-soluble carbohydrates, buffering capacity, and epiphytic microbial populations were not evaluated in the present study, which may limit further interpretation of the fermentation pathways observed in the silages.

This study found that inoculating sorghum silage increased LAB populations, aligning with previous reports (Chotimah et al., 2024; Lee et al., 2021; Paradhipta et al., 2020, 2021). Higher LAB counts in LB and LP silages led to more lactate, creating a low pH that suppressed mould growth (Kung et al., 2018; Muck et al., 2018; Queiroz et al., 2018).

In subtropical regions, silages such as corn, rye, and sorghum remain stable for more than two days (Lee et al., 2019; Paradhipta et al., 2019; Weinberg et al., 2011). In tropical areas, higher temperatures and humidity shorten stability, encouraging spoilage microbes after air exposure (Ardiansyah et al., 2025; Koc et al., 2009; Lee et al., 2019). Temperatures of 30-37°C accelerate deterioration by reducing LAB and promoting yeast, moulds, and CO₂ production (Koc et al., 2009). During this study, ambient temperature averaged 32.3°C in the afternoon and 26.7°C in the evening. LB silage extended shelf-life, linked to higher acetate levels. Heterofermentative LAB in LB silage generate acetate, which inhibits spoilage microbes (Holzer et al., 2003; Danner et al., 2003; Paradhipta et al., 2021). However, excess lactate can reduce stability, as it fuels yeast growth that occurs under aerobic conditions (Danner et al., 2003). Moreover, Danner et al. (2003) reported that yeast and mould use lactate as an energy source for growth during aerobic conditions, which could reduce the aerobic stability of silage.

LAB inoculation increased ruminal digestibility, as LB and LP silages showed higher IVOMD than CON, in line with other studies (Chotimah et al., 2024; Lee, Paradhipta et al., 2021; Paradhipta et al., 2020). LAB may function as probiotics that stimulate cellulolytic

microbes, improving fibre breakdown (Ellis et al., 2016; Oskoueian et al., 2021). However, the present study was based on an *in vitro* ruminal fermentation system and did not directly assess cellulolytic microbial populations or enzyme activities. Therefore, the proposed mechanism should be interpreted with caution, and further *in vivo* studies are needed to confirm whether similar responses occur under practical feeding conditions.

CONCLUSION

In conclusion, the application of both *Lactobacillus brevis* and *Lactobacillus plantarum*, representing hetero- and homofermentative LAB, respectively, improved fermentation quality, reduced nutrient losses and mould growth, and enhanced *in vitro* ruminal digestibility of sorghum silage. However, the use of *Lactobacillus brevis* is more effective, as it provides additional benefits in enhancing the stability of aerobic sorghum silage under tropical situations. In addition, further *in vivo* studies are required to confirm the beneficial effects of hetero- and homofermentative LAB on the nutrient utilisation of animals under practical feeding conditions.

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REFERENCES

- Ardiansyah, M., Fitriani, D., Noviandi, C. T., Kurniawati, A., & Paradhita, D. H. V. (2025). Preservation of high-moisture sorghum silage using combination of biological and chemical additives in the tropical region. *Tropical Animal Science Journal*, 48(3), 257-266. <https://doi.org/10.5398/tasj.2025.48.3.257>
- Arriola, K. G., Kim, S. C., & Adesogan, A. T. (2011). Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. *Journal of Dairy Science*, 94(3), 1511-1516. <https://doi.org/10.3168/jds.2010-3807>
- Association of Official Analytical Chemists. (2005). *Official methods of analysis* (18th ed.). AOAC International.
- Bernardes, T. F., Daniel, J. L. P., Adesogan, A. T., McAllister, T. A., Drouin, P., Nussio, L. G., Huhtanen, P., Tremblay, G. F., Bélanger, G., & Cai, Y. (2018). Silage review: Unique challenges of silages made in hot and cold regions. *Journal of Dairy Science*, 101(5), 4001-4019. <https://doi.org/10.3168/jds.2017-13703>
- Borreani, G., Tabacco, E., Schmidt, R. J., Holmes, B. J., & Muck, R. E. (2018). Silage review: Factors affecting dry matter and quality losses in silages. *Journal of Dairy Science*, 101(5), 3952-3979. <https://doi.org/10.3168/jds.2017-13837>
- Chaney, A. L., & Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry*, 8(2), 130-132. <https://doi.org/10.1093/clinchem/8.2.130>
- Chotimah, Q., Nada, M., Rahayu, E. D., Paradhita, D. H. V., Sanjaya, H. L., Wardani, A. R. D., & Anam, M. S. (2024). Effects of *Achatina fulica* mucus as an antimicrobial additive on chemical compositions,

- fermentation quality, and in vitro digestibility of elephant grass silage. *Veterinary Integrative Sciences*, 22(2), 667-681. <https://doi.org/10.12982/VIS.2024.045>
- Cooke, A. S., Machekano, H., Gwiriri, L. C., Tinsley, J. H. I., Silva, G. M., Nyamukondiwa, C., Safalaoh, A., Morgan, E. R., & Lee, M. R. F. (2025). The nutritional feed gap: Seasonal variations in ruminant nutrition and knowledge gaps in relation to food security in Southern Africa. *Food Security*, 17(1), 73-100. <https://doi.org/10.1007/s12571-024-01509-1>
- Dahlya, D., Zain, M., Agustin, F., Pazla, R., Yanti, G., & Ikhlas, Z. (2025). Rumen fermentation profiles of sorghum-legume mixtures: A strategy for sustainable and efficient ruminant feeding. *International Journal of Veterinary Science*, 14(5), 1047-1055. <https://doi.org/10.47278/journal.ijvs/2025.075>
- Danner, H., Holzer, M., Mayrhuber, E., & Braun, R. (2003). Acetic acid increases stability of silage under aerobic conditions. *Applied and Environmental Microbiology*, 69(1), 562-567. <https://doi.org/10.1128/AEM.69.1.562-567.2003>
- Ellis, J. L., Bannink, A., Hindrichsen, I. K., Kinley, R. D., Pellikaan, W. F., Milora, N., & Dijkstra, J. (2016). The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production. *Animal Feed Science and Technology*, 211, 61-74. <https://doi.org/10.1016/j.anifeedsci.2015.10.016>
- Fitriani, D., Ardiansyah, M., Kurniawati, A., Bachruddin, Z., & Paradhipta, D. H. V. (2024). Chemical and physical quality, fermentation characteristics, aerobic stability, and ruminal degradability of sorghum silage inoculated with *Lactiplantibacillus plantarum* and *Limosilactobacillus fermentum*. *Tropical Animal Science Journal*, 47(4), 483-492. <https://doi.org/10.5398/tasj.2024.47.4.483>
- Holzer, M., Mayrhuber, E., Danner, H., & Braun, R. (2003). The role of *Lactobacillus buchneri* in forage preservation. *Trends in Biotechnology*, 21(6), 282-287. [https://doi.org/10.1016/S0167-7799\(03\)00106-9](https://doi.org/10.1016/S0167-7799(03)00106-9)
- Kim, S. C., & Adesogan, A. T. (2006). Influence of ensiling temperature, simulated rainfall, and delayed sealing on fermentation characteristics and aerobic stability of corn silage. *Journal of Dairy Science*, 89(8), 3122-3132. [https://doi.org/10.3168/jds.S0022-0302\(06\)72586-3](https://doi.org/10.3168/jds.S0022-0302(06)72586-3)
- Kleinschmit, D. H., Schmidt, R. J., & Kung, L. (2005). The effects of various antifungal additives on the fermentation and aerobic stability of corn silage. *Journal of Dairy Science*, 88(6), 2130-2139. [https://doi.org/10.3168/jds.S0022-0302\(05\)72889-7](https://doi.org/10.3168/jds.S0022-0302(05)72889-7)
- Koc, F., Coskuntuna, L., Ozduven, M. L., Coskuntuna, A., & Samli, H. E. (2009). The effects of temperature on the silage microbiology and aerobic stability of corn and vetch-grain silages. *Acta Agriculturae Scandinavica, Section A — Animal Science*, 59(4), 239-246. <https://doi.org/10.1080/09064700903490596>
- Kung, L., Shaver, R. D., Grant, R. J., & Schmidt, R. J. (2018). Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *Journal of Dairy Science*, 101(5), 4020-4033. <https://doi.org/10.3168/jds.2017-13909>
- Lee, S. S., Choi, J. S., Paradhipta, D. H. V., Joo, Y. H., Lee, H. J., Noh, H. T., Kim, D. H., & Kim, S. C. (2021). Application of selected inoculant producing antifungal and fibrinolytic substances on rye silage with different wilting time. *Processes*, 9(5), Article 879. <https://doi.org/10.3390/pr9050879>

- Lee, S. S., Lee, H. J., Paradhipta, D. H. V., Joo, Y. H., Kim, S. B., Kim, D. H., & Kim, S. C. (2019). Temperature and microbial changes of corn silage during aerobic exposure. *Asian-Australasian Journal of Animal Sciences*, 32(7), 988-995. <https://doi.org/10.5713/ajas.18.0566>
- Li, D., Ni, K., Zhang, Y., Lin, Y., & Yang, F. (2019). Fermentation characteristics, chemical composition and microbial community of tropical forage silage under different temperatures. *Asian-Australasian Journal of Animal Sciences*, 32(5), 665-674. <https://doi.org/10.5713/ajas.18.0085>
- Liu, Q., Dong, Z., & Shao, T. (2018). A dinâmica de mudança na fermentação e perfis de ácidos graxos em silagem de alfafa de alta humanidade durante a ensilagem a diferentes temperaturas [The dynamics of change in fermentation and fatty acid profiles in high-moisture alfalfa silage during ensiling at different temperatures]. *Ciência Rural*, 48(3), Article e20170605. <https://doi.org/10.1590/0103-8478cr20170605>
- Muck, R. E., Nadeau, E. M. G., McAllister, T. A., Contreras-Govea, F. E., Santos, M. C., & Kung, L. (2018). Silage review: Recent advances and future uses of silage additives. *Journal of Dairy Science*, 101(5), 3980-4000. <https://doi.org/10.3168/jds.2017-13839>
- Ogunade, I. M., Martinez-Tupia, C., Queiroz, O. C. M., Jiang, Y., Drouin, P., Wu, F., Vyas, D., & Adesogan, A. T. (2018). Silage review: Mycotoxins in silage: Occurrence, effects, prevention, and mitigation. *Journal of Dairy Science*, 101(5), 4034-4059. <https://doi.org/10.3168/jds.2017-13788>
- Oskoueian, E., Jahromi, M. F., Jafari, S., Shakeri, M., Le, H. H., & Ebrahimi, M. (2021). Manipulation of rice straw silage fermentation with different types of lactic acid bacteria inoculant affects rumen microbial fermentation characteristics and methane production. *Veterinary Sciences*, 8(6), Article 100. <https://doi.org/10.3390/vetsci8060100>
- Paradhipta, D. H. V., Hidayah, K. T., Sari, P. C., Firdaus, N., Astuti, A., & Joo, Y. H. (2024). Technical note: Silo type for laboratory scale experiment on the silage quality. *Buletin Peternakan*, 48(3), 187-192. <https://doi.org/10.21059/buletinpeternak.v48i3.95351>
- Paradhipta, D. H. V., Joo, Y. H., Lee, H. J., Lee, S. S., Kim, D. H., Kim, J. D., & Kim, S. C. (2019). Effects of inoculant application on fermentation quality and rumen digestibility of high moisture sorghum-sudangrass silage. *Journal of Applied Animal Research*, 47(1), 486-491. <https://doi.org/10.1080/09712119.2019.1670667>
- Paradhipta, D. H. V., Joo, Y. H., Lee, H. J., Lee, S. S., Noh, H. T., Choi, J. S., Kim, J., Min, H. G., & Kim, S. C. (2021). Effects of inoculants producing antifungal and carboxylesterase activities on corn silage and its shelf life against mould contamination at feed-out phase. *Microorganisms*, 9(3), 1-16, Article 558. <https://doi.org/10.3390/microorganisms9030558>
- Paradhipta, D. H. V., Lee, S. S., Kang, B., Joo, Y. H., Lee, H. J., Lee, Y., Kim, J., & Kim, S. C. (2020). Dual-purpose inoculants and their effects on corn silage. *Microorganisms*, 8(5), Article 765. <https://doi.org/10.3390/microorganisms8050765>
- Pazla, R., Agustin, F., Ikhlas, Z., Ardani, L. R., Sari, A., Cahyana, P. T., Iswari, K., Ardinal, Jumjunidang, Marlina, L., Haloho, J. D., Susanti, I., Syaputri, M., & Fitri, S. Y. (2025). Evaluation of Sonia (BMR mutant sorghum and *Tithonia diversifolia*) usage as sustainable alternative feed to reduce concentrate dependency in ruminant diet: In vitro study. *International Journal of Veterinary Science*, 14(5), 854-859. <https://doi.org/10.47278/journal.ijvs/2025.033>

- Queiroz, O. C. M., Arriola, K. G., Daniel, J. L. P., & Adesogan, A. T. (2013). Effects of 8 chemical and bacterial additives on the quality of corn silage. *Journal of Dairy Science*, 96(9), 5836-5843. <https://doi.org/10.3168/jds.2013-6691>
- Queiroz, O. C. M., Ogunade, I. M., Weinberg, Z., & Adesogan, A. T. (2018). Silage review: Foodborne pathogens in silage and their mitigation by silage additives. *Journal of Dairy Science*, 101(5), 4132-4142. <https://doi.org/10.3168/jds.2017-13901>
- Reddy, Y. R., & Blümmel, M. (2020). Options for enhancing sorghum forage utilisation in ruminants. In V. A. Tonapi, H. S. Talwar, A. K. Are, B. V. Bhat, Ch. R. Reddy, & T. J. Dalton (Eds.), *Sorghum in the 21st century: Food - fodder - feed - fuel for a rapidly changing world* (pp. 667-686). Springer. https://doi.org/10.1007/978-981-15-8249-3_26
- Selim, L., Ataku, K., & Tharwat, M. (2024). Ensilage characteristics of corn silage treated with fermented green juice prepared from corn, alfalfa or timothy. *International Journal of Agriculture and Biosciences*, 13(3), 276-279. <https://doi.org/10.47278/journal.ijab/2024.109>
- Singh, P., Nedumaran, S., Traore, P. C. S., Boote, K. J., Rattunde, H. F. W., Prasad, P. V. V., Singh, N. P., Srinivas, K., & Bantilan, M. C. S. (2014). Quantifying potential benefits of drought and heat tolerance in rainy season sorghum for adapting to climate change. *Agricultural and Forest Meteorology*, 185, 37-48. <https://doi.org/10.1016/j.agrformet.2013.10.012>
- Tilley, J. M. A., & Terry, R. A. (1963). A two-stage technique for the in vitro digestion of forage crops. *Grass and Forage Science*, 18(2), 104-111. <https://doi.org/10.1111/j.1365-2494.1963.tb00335.x>
- Tao, L., Guo, X. S., Zhou, H., Undersander, D. J., & Nandety, A. (2012). Short communication: Characteristics of proteolytic activities of endo- and exopeptidases in alfalfa herbage and their implications for proteolysis in silage. *Journal of Dairy Science*, 95(8), 4591-4595. <https://doi.org/10.3168/jds.2012-5383>
- Vargas, J. A. C., Araújo, T. C. de, & Mezzomo, R. (2020). *A protocol for the extraction, identification, and quantification of short-chain fatty acids (SCFAs) in silages using Reverse Phase - High Performance Liquid Chromatography with Diode Array Detector (RP-HPLC-DAD)* [Data set]. Research Square. <https://doi.org/10.21203/rs.3.pex-1170/v1>
- Weinberg, Z. G., Khanal, P., Yildiz, C., Chen, Y., & Arieli, A. (2011). Ensiling fermentation products and aerobic stability of corn and sorghum silages. *Grassland Science*, 57(1), 46-50. <https://doi.org/10.1111/j.1744-697X.2010.00207.x>
- Reuter, W. M., Elmer, I. P., & Shelton, C. T. (2015). *The analysis of a broad range of organic acids by HPLC with UV detection* (Application Note). PerkinElmer

Review Article

Organic Amendment-Assisted Phytoremediation of Heavy Metals-Contaminated Paddy Soils

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ABSTRACT

Paddy soils are increasingly threatened by heavy metal contamination from the overuse of agrochemicals, industrial emissions, and wastewater irrigation because these challenges pose risks to food safety, crop productivity, and environmental quality. High costs, environmental trade-offs, or low effectiveness under flooded paddy conditions often limit the use of conventional remediation techniques. This review examines the synergistic potential of organic amendment-assisted phytoremediation (OAAP) as a sustainable approach for detoxifying paddy soils contaminated with heavy metals. This approach combines plant-based remediation with the application of organic amendments to enhance the bioavailability of heavy metals, support plant growth and development, and restore soil health. We selected eight studies from the literature published between 2005 and 2025 using strict inclusion criteria. The reviewed studies demonstrate that OAAP significantly improves heavy metals uptake by hyperaccumulator plants by enhancing soil organic matter, microbial activity, and enzymatic functions. The mechanisms identified include chelation, rhizosphere acidification, activation of antioxidative enzymes, and shifts in microbial communities. However, post-remediation concerns such as safe biomass disposal and long-term soil health remain underexplored. Although OAAP demonstrates strong potential in controlled settings, field-scale

validations and comprehensive risk assessments are essential for broader adoption. This review highlights OAAP's promise as a nature-based and low-input strategy for rehabilitating heavy metal-contaminated paddy soils, aligning with the goals of sustainable agriculture.

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INTRODUCTION

Paddy soils are anthropogenic wetland soils developed for rice cultivation in bunded and levelled fields, where the soil is intentionally flooded and puddled under water-saturated conditions (Liu et al., 2021; Witt & Haefele, 2005). As of 2023, paddy cultivation covered approximately 168 million hectares worldwide (Shahbandeh, 2025). This hectareage is the backbone of the global rice production, a cereal crop which serves as a staple food for more than 50% of the world's population (Wang et al., 2024). However, these agroecosystems are increasingly threatened by different pollutants arising from agricultural and anthropogenic activities. Chemical contamination from commercial fertilisers, organic manures, pesticides, herbicides, and untreated wastewater irrigation has significantly degraded paddy soil quality (Nuruzzaman, 2025). This degradation could jeopardise paddy grain yield and food safety and disrupt critical soil functions such as nutrient cycling and microbial balance. Furthermore, polluted paddy soils may increase environmental risks by promoting contaminants' movement into downstream water bodies and into human and animal food chains (Akram et al., 2018).

Among the various pollutants affecting paddy systems, heavy metals stand out because they are persistent and hazardous. For example, cadmium (Cd), arsenic (As), and mercury (Hg), primarily introduced through industrial emissions and the excessive use of agrochemicals, have become a significant concern in paddy farms. At toxic levels, these heavy metals degrade soil health, diminish crop productivity, and compromise food safety (Tang et al., 2020; Wang et al., 2024). Excessive Cd accumulation in rice from contaminated paddy soils may be transferred to the food chain and cause health issues, such as lung cancer and renal tubular dysfunction, thus necessitating its mitigation (Rasin et al., 2025; Wang et al., 2024). Similarly, although arsenic is not an essential element for humans, chronic arsenic intake through rice consumption beyond safe limits damages the kidneys, lungs, and liver (Zheng et al., 2019). Moreover, methylated Hg, which forms under anaerobic soil conditions that are typical of flooded paddy fields, may also bioaccumulate in the food chain, posing health risks including nervous, respiratory, and cardiovascular disorders to humans (Charkiewicz et al., 2025; Zhou et al., 2023).

Paddy soils are vulnerable due to their periodic flooding and fluctuating redox conditions, a process which enhances the mobility and uptake of certain heavy metals by rice plants. These challenges call for effective, sustainable, and locally adaptable remediation strategies to restore paddy soil health in a manner which safeguards food systems. Conventional mitigation strategies include intermittent irrigation, soil passivation using inorganic or organic amendments, phytoremediation, leaching, electrokinetic remediation, and selecting low-accumulation rice cultivars (Chen et al., 2022; Hu et al., 2013; Zhao et al., 2022). Although each of these methods possesses certain advantages, they have some limitations. For example, intermittent irrigation reduces methane emissions, but this practice inadvertently increases As and Cd uptake because of redox-driven changes in heavy metal

solubility (Cui et al., 2025). Soil passivation and phytoremediation are slow or ineffective for composite pollution. Moreover, many conventional approaches lack adaptability to different soil types and climatic conditions, and in some cases, trade-offs arise where alleviating one contaminant exacerbates another (Cui et al., 2025).

Considering the challenges in paddy soil remediation, more sustainable and environmentally friendly approaches are gaining attention, particularly the use of organic amendments and phytoremediation. Although organic amendments, such as compost and biochar, have been widely used to improve soil properties and immobilise contaminants, their effectiveness in enhancing overall heavy metal removal remains limited (Wang et al., 2024; Zhou et al., 2023). Similarly, although phytoremediation is cost-effective and environmentally friendly, its performance is often constrained by the low bioavailability of heavy metals in soils (Tang et al., 2020; Wang, 2024).

To address the afore-stated limitations, the integration of organic amendments with phytoremediation, hereafter referred to as organic amendment-assisted phytoremediation (OAAP), has emerged. Organic amendment-assisted phytoremediation leverages the synergy between organic amendments and plant-based remediation processes, which may include the extraction of heavy metals by hyperaccumulators, microbial stimulation in the rhizosphere, or contaminant immobilisation in the root zone of plants. Through these mechanisms, OAAP can improve heavy metals' bioavailability, support plant growth, and regulate microbial activity to improve remediation efficiency (Li et al., 2020; Tang et al., 2020; Zhou et al., 2023). Previous reviews have commonly examined phytoremediation or organic amendments separately across broad contaminated soil systems (Anggraini et al., 2025; Ghani et al., 2022; Gao et al., 2023; Sharma et al., 2023). In contrast, this review focuses specifically on their integrated use in paddy soils, where flooding and redox fluctuations strongly influence heavy metals mobility, plant uptake, and remediation outcomes (Cui et al., 2025). Its novelty lies in synthesising paddy-specific OAAP evidence on heavy metals removal, soil health restoration, post-remediation soil reuse, biomass fate, and food safety. To this end, this review critically synthesises the literature on OAAP for paddy soil remediation by examining the approaches used, evaluating their reported outcomes, and identifying research gaps that must be addressed to support its practical implementation in sustainable paddy soil management.

MATERIALS AND METHODS

Data Collection and Screening

We conducted this systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines (Page et al., 2021) to ensure a transparent and replicable methodology. We performed a comprehensive search on 23 February 2025 using search engines such as Scopus, ScienceDirect, Google Scholar,

and AGRICOLA, limiting the search to peer-reviewed journal articles published between 2005 and 2025, and including only articles written in English. To capture a wide range of the literature on OAAP in paddy soils, general and specific search terms were used. These included “organic amendment assisted phytoremediation paddy” and Boolean combinations such as “organic AND amendment AND assisted AND phytoremediation AND paddy.” We also conducted targeted searches using amendment-specific phrases, including “biochar assisted phytoremediation AND paddy” and “compost assisted phytoremediation AND paddy.”

The initial search yielded 461 records. After removing 33 duplicates, we retained 428 unique records for screening. Title and abstract screening resulted in the exclusion of 407 studies that did not meet the review criteria, primarily because of a lack of relevance to OAAP or a focus outside of paddy soil systems. The remaining full-text articles were assessed against predefined inclusion and exclusion criteria, detailed in the following section. The whole process of identification, screening, and selection is summarised in Figure 1.

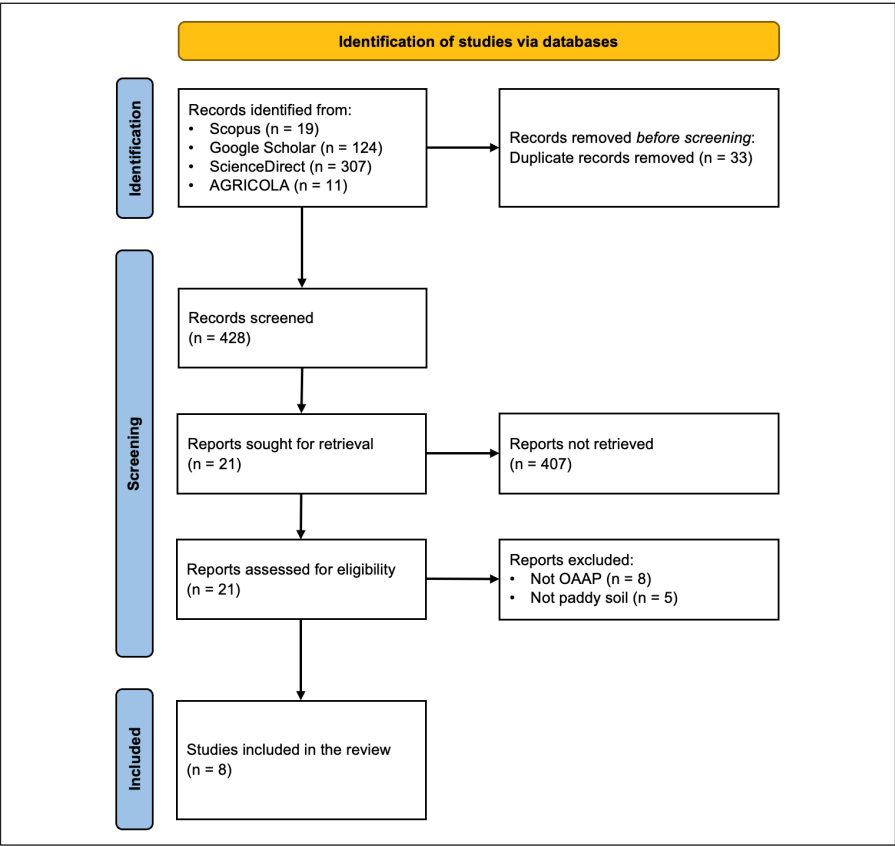


Figure 1. An amended PRISMA 2020 flow diagram illustrating the identification, screening, and inclusion process for studies on organic amendment-assisted phytoremediation (OAAP) in paddy soils

Inclusion and Exclusion Criteria

To determine eligibility for this review, three inclusion criteria were applied. Firstly, studies which focus specifically on the remediation of paddy soils. Secondly, studies which explicitly integrated both organic amendments and phytoremediation were considered. Thirdly, eligible studies were required to assess outcomes such as contaminant reduction, plant heavy metal uptake, or soil quality improvement resulting from the combined treatment. Following title and abstract screening, we reviewed 21 full-text articles and excluded 11 full-text articles because they investigated only one OAAP component (Either organic amendment or phytoremediation), using non-organic amendments, or focusing on non-paddy soil systems. Ultimately, eight studies fulfilled all inclusion criteria and were selected for in-depth analysis. The selected eight studies are summarised in Table 1, which presents the source journal, the number of relevant articles from each journal, and the titles of the included publications.

Table 1
Source journals and titles of articles included in the systematic review on organic amendment-assisted phytoremediation in paddy soils

Journal	No. of Articles	Article Title	Authors
Environmental Science and Pollution Research	1	Biochar-assisted phytoextraction of arsenic in soil using <i>Pteris vittata</i> L.	Zheng et al. (2019)
Environmental Technology & Innovation	1	Biochar assists phytoremediation of cadmium by regulation of rhizosphere microbiome in paddy fields	Wang et al. (2024)
International Journal of Phytoremediation	2	Phytoextraction of copper from contaminated soil by <i>Elsholtzia splendens</i> as affected by EDTA, citric acid, and compost	Yang et al. (2005)
		Impacts of bamboo biochar on the phytoremediation potential of <i>Salix psammophila</i> grown in multi-metal contaminated soil	Li et al. (2020)
Journal of Environmental Management	1	Application potential of biofertiliser-assisted <i>Pennisetum giganteum</i> in safe utilisation of mercury-contaminated paddy fields	Zhou et al. (2023)
Journal of Environmental Science and Health	1	Copper phytoavailability and uptake by <i>Elsholtzia splendens</i> from contaminated soil as affected by soil amendments	Peng et al. (2005)
Plant Soil	1	The efficiency of Cd phytoextraction by <i>S. plumbizincicola</i> increased with the addition of rice straw to polluted soils: the role of particulate organic matter	Zhou et al. (2018)
Sustainability	1	Biochelator-assisted phytoremediation for cadmium (Cd) pollution in paddy fields	Wang et al. (2021)

Data Analysis

The selected studies were analysed qualitatively to extract relevant information on OAAP in paddy soils. For each study, key data were compiled, including the source journal, article title, publication year, and primary research focus. Additional information was recorded on the type of organic amendment used, the phytoremediator species employed, the targeted heavy metal contaminants, and the reported outcomes related to metal uptake, contaminant removal, and improvements in soil quality. Data were organised to compare the interaction between amendment type and phytoremediator, the effectiveness of the remediation approach, and any effects on soil properties or metal bioavailability. Studies that discussed the role of chemical, biological, or physiological mechanisms in enhancing phytoremediation were also noted for later discussion. Due to variability in experimental designs, soil types, contaminant profiles, and data reporting formats, a quantitative meta-analysis was not conducted. Instead, a narrative synthesis approach was used to highlight shared findings, differences, and methodological gaps. The results are presented and subsequently discussed in detail, supported by a summary table (Table 2) that outlines the key findings of each study.

RESULTS AND DISCUSSION

Organic Amendment-assisted Phytoremediation Approaches in Paddy Soils

Among the different phytoremediation approaches applied in paddy soils, phytoextraction was the most frequently adopted approach in the reviewed studies. This process involves the use of hyperaccumulator plants, which are capable of absorbing soluble heavy metals through their roots and translocating them to aboveground tissues, which are later harvested for contaminant removal. Phytoextraction is valued for its simplicity, cost-effectiveness, and potential for biomass valorisation, particularly when combined with organic amendments that enhance heavy metal availability and plant health (Awad et al., 2021; Li et al., 2020).

Several combinations of organic amendments and hyperaccumulator species have been used to remediate heavy metal-contaminated paddy soils. We classified the organic amendments used into five categories: agricultural wastes, composts, biochars, biochelators, and biofertilisers. Table 2 summarises these approaches by outlining the type of organic amendment, the phytoremediator, the target pollutant, and the key findings, including improvements in heavy metal uptake, plant performance, and soil conditions. Altogether, these combinations reflect the diversity and adaptability of OAAP approaches across different paddy soil systems.

Table 2
Summary of key findings from the selected papers on organic amendment-assisted phytoremediation of paddy soils contaminated with heavy metals

No.	Organic Amendment	Phytoremediator	Pollutant	Key Findings	Reference
1	Compost	<i>Elsholtzia splendens</i>	Copper (Cu)	Increased water-extractable Cu by shifting Cu into more bioavailable exchangeable and organic-bound forms. Resulted in a 3.6-fold increase in Cu extraction. Enhanced shoot Cu accumulation in <i>E. splendens</i> , especially in paddy soils. Compost was more effective in paddy soils than in mined soils. Demonstrated suitability of <i>E. splendens</i> for Cu phytoextraction when paired with compost.	Yang et al. (2005)
2	Manure	<i>Elsholtzia splendens</i>	Cu	Increased water-exchangeable Cu in contaminated paddy soil. Stimulated Cu uptake and shoot biomass in <i>E. splendens</i> . Mechanisms involved rhizospheric acidification and DOM chelation. Highlighted the Cu tolerance, high biomass, and accumulation potential of <i>E. splendens</i> . Manure at appropriate rates significantly improved Cu bioavailability and phytoextraction efficiency.	Peng et al. (2005)
3	Rice straw	<i>Sedum plumbizincicola</i>	Cd	Increased Cd uptake by 14.3-20.7% in the first harvest, and this positive effect was sustained across four cropping cycles. Significantly raised soil POM mass. Enhanced oxidizable Cd fractions and Cd bioavailability within POM pools. Improved availability of N, P, and K, supporting plant growth and biomass production. Cd concentrations in POM fractions decreased after repeated phytoextraction.	Zhou et al. (2018)

Table 2 (continued)

No.	Organic Amendment	Phytoremediator	Pollutant	Key Findings	Reference
4	Rice straw-derived biochar	<i>Pteris vittata</i> L.	As	Alkaline biochar raised soil pH, mobilising As and increasing its bioavailability. Biochar amendment (1-5%) significantly enhanced As accumulation in <i>P. vittata</i> after 40 days. DGT measurements indicated active root uptake and spatial As redistribution. Rates >1% posed a risk of As leaching, highlighting the need for rate optimisation. Biochar used had negligible As content, preventing secondary contamination.	Zheng et al. (2019)
5	Bamboo-derived biochar	<i>Salix psammophila</i>	Cd, Zinc (Zn), Cu, and lead (Pb)	Increased soil organic matter and nutrient availability yet reduced bioavailable metal fractions. Enhanced accumulation of Cd, Zn, and Cu in <i>S. psammophila</i> . Optimal BBC dose (1-5%) supported plant growth; 7% suppressed biomass. Improved bioconcentration and translocation factors for Cd and Zn. Demonstrated potential for cost-effective, eco-friendly in situ remediation using fast-growing willow species.	Li et al. (2020)
6	Biochelators (Humic acid and rhamnolipid)	<i>Iris sibirica</i> L.	Cd	HA and RL treatments improved growth, Cd uptake, and stress tolerance in <i>I. sibirica</i> under Cd-contaminated conditions. HA increased dry weights of leaves (3 mg L ⁻¹), stems (7 mg L ⁻¹), and roots at optimal doses. HA reduced Cd accumulation in plant tissues, indicating immobilisation. RL enhanced Cd accumulation in stems and roots, indicating increased phytoavailability. RL also elevated SOD, POD, and CAT activities, improving antioxidative stress response.	Wang et al. (2021)

Table 2 (continued)

No.	Organic Amendment	Phytoremediator	Pollutant	Key Findings	Reference
7	Biofertiliser (<i>Bacillus</i> -inoculated distiller's grain)	<i>Pennisetum giganteum</i>	Hg	Inoculation with Hg-resistant <i>Bacillus</i> sp. increased soil <i>Bacillus</i> abundance by 157.12%. Enabled removal of 27.52% of water-soluble Hg <i>via</i> microbial volatilisation and adsorption. Reduced THg in plant tissues by 48-94%, depending on organ. Significantly lowered MeHg concentrations across plant parts. Biofertiliser-assisted <i>P. giganteum</i> cultivation generated fourfold higher income than traditional rice planting.	Zhou et al. (2023)
8	Moss-derived biochar	<i>Solanum nigrum</i> and <i>Echinochloa crus-galli</i>	Cd	Porous biochar structure (~4.79 nm) provided binding sites and habitat for beneficial microbes. Improved Cd removal rate from 11-15% to 23-35% after 60 days. Increased bacterial abundance from ~2.26-2.76 × 10 ⁶ to 3.89-4.78 × 10 ⁶ cells g ⁻¹ soil. Enhanced organic matter and enzyme activities, supporting soil fertility and plant health. Enriched stress-tolerant, biofilm-forming bacteria that remodelled the rhizosphere and improved Cd tolerance.	Wang et al. (2024)

Note. DOM = dissolved organic matter; POM = particulate organic matter; DGT = diffusive gradients in thin films; HA = humic acid; RL = rhamnolipid; SOD = superoxide dismutase; POD = peroxidase; CAT = catalase; THg = total mercury; MeHg = methylmercury

Mechanisms Underpinning Organic Amendment-Assisted Phytoremediation

Organic amendments influence the mobility of heavy metals, plant uptake, and soil functionality through a combination of chemical, biological, and physiological processes. These mechanisms play a role in enhancing the bioavailability of target heavy metals, thereby improving plant health and soil microbial dynamics, and ultimately facilitating more efficient and sustainable phytoremediation. The key mechanisms identified in the reviewed studies are subsequently discussed based on their contributions to the availability of heavy metals, plant performance, and soil restoration (Figure 2).

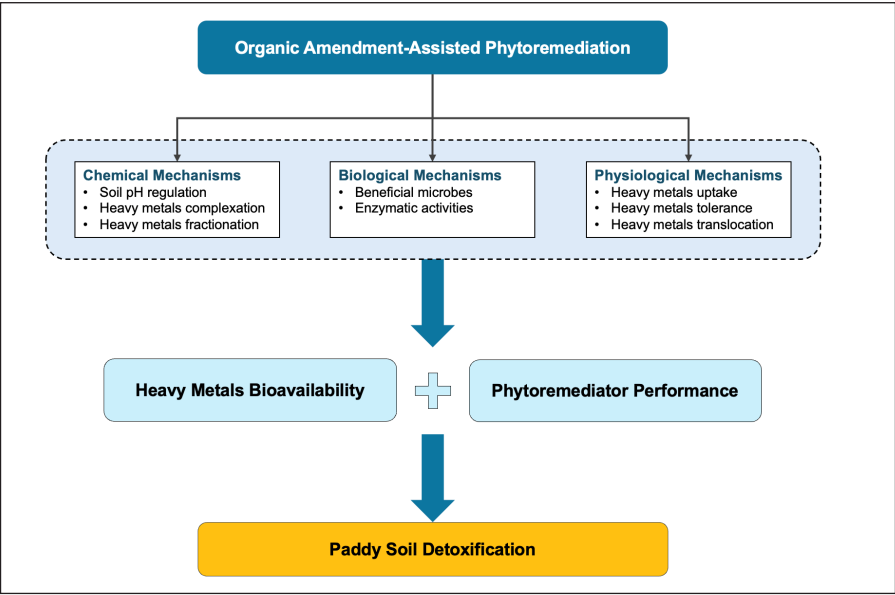


Figure 2. A summary of the three primary mechanisms governing organic amendment-assisted phytoremediation for detoxifying heavy metals-contaminated paddy soils

Enhancement of Heavy Metal Bioavailability

Organic amendments influence the speciation, solubility, and distribution of heavy metals in paddy soils, thereby directly affecting their bioavailability to plants. For example, Yang et al. (2005) found that the application of compost (5% w/w) significantly increased the water-extractable and exchangeable fractions of Cu, resulting in a 3.6-fold increase in Cu phytoextraction by *Elsholtzia splendens*. Similarly, organic manure enhanced Cu bioavailability by stimulating rhizospheric acidification and promoting chelation with dissolved organic matter, both of which increased Cu mobility and uptake (Peng et al., 2005). These effects are attributed to the presence of functional groups such as carboxyl and hydroxyl moieties on organic matter surfaces, which complex with metal ions and shift them into more available fractions (Sabir et al., 2015; Wiszniewska et al., 2016).

For Cd, rice straw amendment elevated particulate organic matter pools and increased oxidizable Cd fractions. Thus, maintaining high Cd bioavailability and supporting uptake by *Sedum plumbizincicola* across four cropping cycles (Zhou et al., 2018). In As-contaminated soils, rice straw-derived biochar increased soil pH, and this shifts As into more labile forms, enhancing As accumulation in *Pteris vittata*. However, higher application rates (>1%) pose a potential leaching risk (Zheng et al., 2019). Likewise, bamboo-derived biochar (BBC) demonstrated a dual role, namely reducing the soluble fractions of Cd, Zn, Cu, and Pb, yet supporting their accumulation in *Salix psammophila* through improved

bioconcentration and translocation (Li et al., 2020). These findings demonstrate that whereas some amendments mobilise heavy metals to facilitate uptake, others immobilise excess bioavailable fractions to reduce phytotoxicity. Hence, suggesting the importance of both dose and metal type.

Further evidence demonstrates that biochelators, such as humic acids (HAs) and rhamnolipid (RL), modulate heavy metal bioavailability in contrasting ways. Humic acids reduced Cd uptake in *Iris sibirica* by immobilising free Cd ions in the rhizosphere, whereas RL increased Cd accumulation in stems and roots, likely by enhancing Cd solubility (Wang et al., 2021). These outcomes illustrate how the same amendment class can induce opposing effects depending on formulation and target heavy metal, thus underscoring the context-specific nature of OAAP strategies. Overall, OAAP-enhanced heavy metals bioavailability is governed by mechanisms such as pH regulation, heavy metal complexation, chelation, and redistribution of heavy metal fractions, all of which vary with the type and composition of the organic amendment used.

Improvement of Phytoremediator Plant Performance

Organic amendments play a pivotal role in enhancing plant performance under heavy metal stress. This is essential for successful phytoremediation. The reviewed studies reported improvements in biomass, heavy metal translocation efficiency, nutrient uptake, and stress tolerance across various OAAP systems. For example, *Elsholtzia splendens*, which is reputed for its high tolerance to Cu and biomass, exhibited significantly enhanced growth and Cu accumulation when treated with compost or organic manure, particularly through improved nutrient availability and reduced metal toxicity (Peng et al., 2005; Yang et al., 2005). In a long-term study, rice straw amendment supported biomass production and sustained Cd uptake in *Sedum plumbizincicola* over four consecutive cropping cycles, demonstrating the potential of OAAP to deliver durable physiological improvements (Zhou et al., 2018). Similarly, BBC improved the accumulation of Cd, Zn, and Cu in *Salix psammophila* through enhancing bioconcentration and translocation factors. However, excessive BBC application (7%) suppresses plant growth, highlighting the importance of optimising amendment dosage (Li et al., 2020). In *Pteris vittata*, rice straw-derived biochar increased As translocation to aboveground parts through increasing soil pH and enhancing uptake mechanisms (Zheng et al., 2019).

Beyond uptake and biomass, OAAP enhances the phytoremediator plant's tolerance to oxidative stress. In *Iris sibirica*, the HAs application improved nutrient uptake and increased the dry weights of leaves, stems, and roots, whereas the RL treatment activated antioxidant defence enzymes, such as superoxide dismutase, peroxidase, and catalase, and this enables the plant to cope with Cd-induced stress (Wang et al., 2021). These findings suggest that organic amendments can modulate physiological responses such as photosynthesis, root

growth, metal transport, and defence enzyme activities, all of which contribute to enhanced remediation performance.

In a field application of biofertiliser derived from *Bacillus*-inoculated distiller's grains, *Pennisetum giganteum* exhibited vigorous growth and a marked reduction in Hg accumulation in the plant tissues (Zhou et al., 2023). This biofertiliser-assisted *P. giganteum* cultivation system generated economic returns, approximately four times higher than those from traditional rice cultivation on Hg-contaminated paddy fields, reflecting both improved physiological health and enhanced agronomic viability (Zhou et al., 2023). Generally, these studies demonstrate that organic amendments enhance plant performance by mitigating heavy metal toxicity, reducing phytotoxic stress, stimulating growth parameters, and promoting metal translocation, which are key attributes that underpin the effectiveness of phytoremediation.

Restoration of Paddy Soil Health

Besides facilitating the removal of heavy metals and promoting plant growth, organic amendments play a crucial role in restoring soil health through enhancing microbial dynamics, enzymatic activities, organic matter content, and nutrient cycling. These biological improvements are essential for maintaining remediation outcomes and restoring long-term soil functionality in paddy cultivation. For example, the application of moss-derived biochar increased soil organic matter, microbial biomass, and enzyme activities, such as urease, acid phosphatase, and catalase. These are among the indicators of improved soil fertility and biological function (Wang et al., 2024). The same study reported a significant increase in rhizosphere bacterial populations, from approximately $2.26\text{--}2.76 \times 10^6$ to $3.89\text{--}4.78 \times 10^6$ cells g^{-1} of soil, alongside an enrichment of stress-tolerant and biofilm-forming bacteria that remodelled the rhizosphere and promoted Cd tolerance and removal. Similarly, the use of a biofertiliser comprising *Bacillus*-inoculated distiller's grains significantly increased the abundance of Hg-resistant *Bacillus* spp. It enabled microbial volatilisation and adsorption of Hg, resulting in reduced Hg accumulation in *Pennisetum giganteum* tissues (Zhou et al., 2023). These biological activities may reduce pollutant load and contribute to soil microbial reconditioning, an essential factor in post-remediation resilience.

Broader studies have also revealed that organic input such as compost, manure, and biochar foster shifts in microbial community structure, favouring taxa that are capable of either solubilising metals to support phytoextraction or immobilising them through transformation processes (Duan et al., 2022; Hussain et al., 2021; Wiszniewska et al., 2016). In several cases, enhanced microbial activity coincided with improved soil enzyme activities and better root-soil interactions, indirectly supporting plant health and remediation efficiency. Collectively, studies reviewed in this present paper highlight that

OAAP contributes to contaminant reduction, alongside the biological recovery of soil systems through enhanced microbial functioning, organic matter enrichment, and improved biochemical processes, thereby supporting sustainable land use post-remediation.

Safe Use of Treated Paddy Soils

Successful remediation extends beyond contaminant removal; it must also ensure that the remediated paddy soil is safe for sustainable rice production, especially in systems where soil quality directly influences rice safety and consequently, regional food security. Although OAAP has shown promising results in lowering extractable heavy metal fractions and improving soil fertility, the long-term safety of planting edible crops, particularly rice, on remediated soils remains uncertain. For example, Wang et al. (2024) demonstrated that moss biochar-assisted phytoremediation significantly reduced soil Cd concentrations and improved enzyme activity and organic matter content, indicating enhanced soil quality. However, the study did not evaluate the implications of these changes on paddy productivity or rice grain heavy metal content. Similarly, Zhou et al. (2023) reported successful Hg reduction in paddy soils using biofertiliser-assisted *Pennisetum giganteum*, but the reuse potential of the remediated soil for food crop cultivation was not assessed. These findings highlight a recurring limitation in OAAP research: although heavy metal extraction efficiency is well-documented, the post-remediation suitability of soils for food production, especially in terms of residual bioavailable heavy metal fractions and food safety thresholds, remains underexplored.

The study by Tamma et al. (2025) revealed limited focus on the post-remediation suitability of soils for food crop production, underscoring the importance of moving beyond contaminant removal to address the long-term usability of the remediated soils. Although some OAAP strategies have demonstrated effectiveness in reducing total heavy metal concentrations and enhancing soil quality, many studies neglect the issue of whether these improvements translate into safe conditions for growing food crops such as rice. This is important because the residual bioavailable heavy metals may still threaten crop safety, particularly when left unassessed. This is problematic in paddy systems, where heavy metal uptake by rice plants can lead to food chain contamination. Tamma et al. (2025) argue that evaluating soil recovery based solely on reduced metal content or improved fertility is insufficient. Instead, they recommend a more rigorous assessment framework that includes testing for plant-available metal fractions, potential for heavy metal re-accumulation in edible tissues, and alignment with established food safety benchmarks. Furthermore, it is also essential to integrate such assessments into national guidelines to support safe agricultural reuse and protect human health. By broadening the scope of OAAP evaluations, future applications can better ensure that detoxified soils are genuinely fit for sustainable food production, especially in regions where rice is a dietary staple and a cornerstone of

food security. Hence, comprehensive post-remediation evaluations, including assessments of heavy metal bioavailability, re-accumulation risk, and crop safety, are needed to support the long-term viability of OAAP in food systems in ensuring healthy crop growth without compromising food quality.

Fate of Post-Remediation Biomass

Evaluating the post-remediation fate involves assessing residual heavy metal concentrations and potential risks of recontamination. Hyperaccumulator biomass harvested after phytoextraction may contain elevated levels of heavy metals, thus making disposal or reuse a potential environmental concern. Therefore, different routes such as added value products, biofuels, composting under monitored conditions, or regulated disposal must be considered to prevent secondary contamination.

The harvested biomass from hyperaccumulator plants represents a critical post-remediation concern, particularly when edible or fast-growing species are used. These plants often contain high levels of accumulated heavy metals in their aerial parts, rendering them unsuitable for direct consumption or composting without proper safeguards. Wang et al. (2021) found that *Iris sibirica* L. accumulated over 1000 mg kg⁻¹ of Cd in roots and stems when assisted by rhamnolipid, a level exceeding the Tolerable Weekly Intake for adults, which is 0.4-0.5 mg per week, or 60-70 µg per day, as recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Charkiewicz et al., 2023). Similarly, Zhou et al. (2023) observed substantial Hg concentrations in *Pennisetum giganteum* tissues after biofertiliser-assisted remediation, including 93.72% reduction in stem total Hg, indicating that biomass management must be approached with caution. One valorisation option is biochar production, which may stabilise heavy metal contents to enable the material to be reused in non-edible applications. However, even biochar must comply with the maximum permissible concentration (MPC) limits for heavy metals before being applied to soil. Table 3 presents the MPC values for several heavy metals

Table 3
Maximum permissible concentration (MPC) for selected heavy metals in paddy soils

Heavy Metal	MPC (mg kg ⁻¹)	Country	Reference
Hg	1.0	China	Du et al. (2021)
Cd	1.5	China	Ji et al. (2012)
As	14	India	Mandal et al. (2023)
Pb	50	China	Wang et al. (2023)
Cu	100	Pakistan	Khan et al. (2024)
Zn	200	Pakistan	Khan et al. (2024)

(As, Cd, Hg, Pb) which threaten paddy plants and food safety when their concentrations exceed the allowable limit, including micronutrients such as Cu and Zn, which become phytotoxic at elevated levels. Alternatively, Ionata et al. (2024) suggest that biomass derived from phytoremediation can be valorised into renewable biofuels, such as bioethanol, biodiesel, and biogas, through biochemical and thermochemical conversion processes. For example, *Pteris vittata* biomass has been utilised as a feedstock for bioethanol and biogas production following As phytoremediation. The biomass was pretreated with ultrapure water and sodium hydroxide, and a subsequent ethanol-assisted anaerobic digestion which enables recovery of energy-rich products while managing As-containing residues.

Ultimately, the responsible handling of post-remediation biomass is important to safeguard environmental and food system integrity. Whether through valorisation into safe, non-edible applications or carefully regulated disposal, post-remediation strategies must ensure that the benefits of phytoremediation are not undermined by unintended pathways of heavy metal re-entry into the ecosystem.

Comparative Sustainability and Economic Viability of Organic Amendment-assisted Phytoremediation and Conventional Remediation Strategies

Organic amendment-assisted phytoremediation aligns well with the goals of sustainable agriculture by promoting waste reutilisation, improving soil health, and reducing environmental risks associated with conventional remediation practices. For example, rice straw, which is often burned in paddy-producing regions, was repurposed as an organic amendment to enhance Cd uptake by *Sedum plumbizincicola*, demonstrating how crop residues can be converted into valuable remediation inputs (Zhou et al., 2018). Similarly, the use of manure and compost supports nutrient recycling and minimises synthetic fertiliser dependence (Peng et al., 2005; Yang et al., 2005). Meanwhile, biochar application improves long-term soil structure, water retention, and carbon sequestration potential (Li et al., 2020; Wang et al., 2024; Zheng et al., 2019). Moreover, the integration of biochelators and biofertilisers further enhances microbial diversity and rhizosphere health, promoting a self-reinforcing system of soil regeneration (Wang et al., 2021; Zhou et al., 2023). These attributes make OAAP a nature-based, low-input alternative to conventional lime-based treatments, which often require repeated application and can lead to long-term soil degradation if mismanaged.

Compared with conventional remediation technologies, OAAP provides a broader sustainability function by combining contaminant removal with soil health restoration. Conventional approaches such as water management, passivation, and low-accumulation rice cultivars can reduce metal mobility, crop uptake, or exposure risk, but they may not necessarily restore contaminated paddy soils through contaminant removal and biological soil recovery. For instance, water management regulates As and Cd availability through

redox changes, but its effects are contaminant-specific: flooding may reduce Cd availability through sulfide precipitation, whereas drying may reduce As availability but increase Cd release through CdS oxidation, making simultaneous As and Cd control difficult under field conditions (Cui et al., 2025). Passivation can effectively reduce metal bioavailability and grain accumulation, as shown by Zhao et al. (2022), who reported lower DTPA-extractable Cd, Cr, and Pb in soil and reduced metal accumulation in rice grains after applying multi-component passivators. However, passivation immobilises metals rather than removing them. Furthermore, low-accumulation rice cultivars represent a food-chain risk mitigation strategy instead of a soil remediation method. They can reduce heavy metals transfer into rice grains, but they mainly manage plant uptake and grain accumulation instead of treating the contaminated soil itself (Hu et al., 2013). Phytoremediation alone is environmentally friendly, but its performance is often limited by low metal bioavailability, slow removal rates, and poor growth of non-hyperaccumulators under metal stress; these limitations explain why assisted strategies using organic amendments have been explored to improve plant-based remediation (Tang et al., 2020; Wyszniowska et al., 2016).

In contrast, OAAP integrates plant-based metal uptake with organic amendment-driven improvements in nutrient supply, microbial activity, organic matter status, and soil biochemical functioning. Several reviewed studies reported measurable improvements in metal uptake, contaminant removal, phytoremediator performance, and soil biological activity under OAAP treatments (Wang et al., 2024; Yang et al., 2005; Zhou et al., 2018; Zhou et al., 2023). However, these outcomes cannot be directly normalised against the conventional methods because the studies differed in contaminant type, remediation endpoint, plant species, amendment type, and experimental duration. Therefore, OAAP is viewed as a soil-restorative strategy that can complement conventional remediation technologies where long-term sustainability and practical affordability are priorities.

From an economic perspective, the use of readily available agricultural wastes such as manure, rice straw, and biochar feedstocks reduces material costs, making OAAP more accessible to resource-limited farming communities. For example, Zhou et al. (2023) reported that biofertiliser-assisted *Pennisetum giganteum* cultivation remediated Hg-contaminated paddy soil and generated income four times higher than traditional rice farming on the same land, highlighting the dual benefits of land rehabilitation and biomass utilisation. Despite these advantages, certain limitations must be addressed. For example, labour-intensive biomass management, variability in amendment quality, and the lack of standardised application guidelines may limit scalability. Additionally, upfront investment in amendment production, such as biochar kilns or composting infrastructure, could pose a barrier for smallholder farmers without institutional support. To this end, comprehensive cost-benefit analyses and policy incentives will be essential to establish OAAP as a viable alternative for large-scale adoption in paddy systems.

Implications and Future Directions

The findings of this present review highlight the potential of OAAP as a sustainable approach for remediating heavy metal-contaminated paddy soils. The studies elucidate that organic amendments can enhance heavy metal uptake by hyperaccumulator plants, besides contributing to long-term improvements in soil quality and microbial health. These dual benefits position OAAP as a nature-based solution that aligns with the goals of sustainable agriculture and circular economy frameworks. Furthermore, the successful use of locally available organic wastes such as rice straw, manure, and compost underscores their practicality in resource-constrained agricultural systems. As paddy cultivation remains central to food security in many countries, the adoption of OAAP strategies may serve as an important step toward improving degraded soils, besides minimising environmental impacts.

However, several gaps and limitations in current OAAP research were also identified. Most of the studies were conducted at pot-scale or under controlled conditions, limiting their applicability to field scenarios. Long-term impacts, crop productivity outcomes, and the potential for residual heavy metal accumulation in subsequent cropping cycles remain underexplored. Moreover, the post-remediation management of phytoremediation biomass, particularly for species that accumulate substantial heavy metal concentrations, requires further investigation to ensure that these materials do not become secondary sources of contamination. This is especially relevant for species such as *Solanum nigrum* and *Pennisetum giganteum*, which are known to be used as food or forage in certain contexts. The potential for heavy metals to enter the food chain *via* consumption or animal feed poses significant health and environmental risks if biomass is not properly evaluated and managed. In addition, mechanisms involving soil-plant-microbe interactions under fluctuating field conditions such as flooding, drainage, or pH shifts are still poorly understood. Future studies should prioritise field-scale validation, multi-season trials, and comprehensive assessments of both agronomic outcomes and environmental safety, to advance OAAP toward practical, large-scale implementation.

CONCLUSION

Organic amendment-assisted phytoremediation presents a sustainable approach for remediating heavy metal-contaminated paddy soils, besides improving soil health and fertility. This review demonstrates that integrating organic amendments with hyperaccumulator plants can enhance heavy metals bioavailability, promote plant growth, and support beneficial microbial activity, which are factors that are essential for effective phytoremediation. However, the success of OAAP is site-specific, influenced by soil properties, contaminant types, and amendment selection. In spite of the encouraging outcomes, the limited number of studies, most of which are pot-based, restricts the

generalizability of current findings. Current studies rarely examine long-term soil health, paddy productivity, and food safety. Moreover, the post-remediation fate of contaminated biomass and the economic feasibility of OAAP require further investigation. Future research should prioritise field-scale validation, assess multi-season impacts, and develop standardised procedures for amendment application and biomass management. Addressing these gaps will be essential to advancing OAAP as a viable, scalable solution for sustainable paddy soil management.

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DECLARATION ON THE USE OF AI-ASSISTED TOOLS

The authors declare that the manuscript was prepared by the authors. The conception of the review, literature search, article screening, data interpretation, critical synthesis, conclusions, and final approval of the manuscript were undertaken by the authors. Artificial Intelligence-assisted tools were used only for language polishing, grammar checking, and improving sentence clarity.

REFERENCES

- Akram, R., Turan, V., Wahid, A., Ijaz, M., Shahid, M. A., Kaleem, S., Hafeez, A., Maqbool, M. M., Chaudhary, H. J., Munis, M. F. H., Mubeen, M., Sadiq, N., Murtaza, R., Kazmi, D. H., Ali, S., Khan, N., Sultana, S. R., Fahad, S., Amin, A., & Nasim, W. (2018). Paddy land pollutants and their role in climate change. *Soil Biology*, 53, 113-124. https://doi.org/10.1007/978-3-319-93671-0_7
- Anggraini, Z., Nurliati, G., Pratama, H. A., Sriwahyuni, H., Sumarbagiono, R., Shadrina, N., Mirawaty, M., Pamungkas, N. S., Putra, Z. P., & Yusuf, M. (2025). A critical review about phytoremediation of heavy metals and radionuclides: From mechanisms to post-remediation strategies. *Chemosphere*, 381, Article 144475. <https://doi.org/10.1016/j.chemosphere.2025.144475>
- Awad, M., El-Desoky, M. A., Ghallab, A., Kubes, J., Abdel-Mawly, S. E., Danish, S., Ratnasekera, D., Islam, M. S., Skalicky, M., Brestic, M., Baazeem, A., Alotaibi, S. S., Javed, T., Shabbir, R., Fahad, S., Rahman, M. H. U., & Sabagh, A. E. (2021). Ornamental plant efficiency for heavy metals phytoextraction from contaminated soils amended with organic materials. *Molecules*, 26(11), Article 3360. <https://doi.org/10.3390/molecules26113360>

- Charkiewicz, A. E., Omeljaniuk, W. J., Garley, M., & Nikliński, J. (2025). Mercury exposure and health effects: What do we really know? *International Journal of Molecular Sciences*, 26(5), Article 2326. <https://doi.org/10.3390/ijms26052326>
- Charkiewicz, A. E., Omeljaniuk, W. J., Nowak, K., Garley, M., & Nikliński, J. (2023). Cadmium toxicity and health effects—A brief summary. *Molecules*, 28(18), Article 6620. <https://doi.org/10.3390/molecules28186620>
- Chen, K., Yu, S., Ma, T., Ding, J., He, P., Li, Y., Dai, Y., & Zeng, G. (2022). Modelling the water and nitrogen management practices in paddy fields with HYDRUS-1D. *Agriculture*, 12(7), Article 924. <https://doi.org/10.3390/agriculture12070924>
- Cui, H., Chen, B., Yang, F., Han, T., Zeng, R., Lei, L., & Liu, S. (2025). A review of research progress on prevention and control technologies for arsenic and cadmium composite pollution in paddy soil. *Environmental Science Advances*, 4(3), 571-583. <https://doi.org/10.1039/D4VA00293H>
- Du, J., Liu, F., Zhao, L., Liu, C., Fu, Z., & Teng, Y. (2021). Mercury horizontal spatial distribution in paddy field and accumulation of mercury in rice as well as their influencing factors in a typical mining area of Tongren City, Guizhou, China. *Journal of Environmental Health Science and Engineering*, 19(2), 1555-1567. <https://doi.org/10.1007/s40201-021-00711-z>
- Duan, Y., Li, Q., Zhang, L., Huang, Z., Zhao, Z., Zhao, H., Du, J., & Zhou, J. (2022). Toxic metals in a paddy field system: A review. *Toxics*, 10(5), Article 249. <https://doi.org/10.3390/toxics10050249>
- Gao, J., Han, H., Gao, C., Wang, Y., Dong, B., & Xu, Z. (2023). Organic amendments for in situ immobilisation of heavy metals in soil: A review. *Chemosphere*, 335, Article 139088. <https://doi.org/10.1016/j.chemosphere.2023.139088>
- Ghani, J., Nawab, J., Khan, S., Khan, M. A., Ahmad, I., Ali, H. M., Siddiqui, M. H., Funari, V., & Dinelli, E. (2022). Organic amendments minimise the migration of potentially toxic elements in soil-plant system in degraded agricultural lands. *Biomass Conversion and Biorefinery*, 14(5), 6547-6565. <https://doi.org/10.1007/s13399-022-02816-3>
- Hu, P., Huang, J., Ouyang, Y., Wu, L., Song, J., Wang, S., Li, Z., Han, C., Zhou, L., Huang, Y., Luo, Y., & Christie, P. (2013). Water management affects arsenic and cadmium accumulation in different rice cultivars. *Environmental Geochemistry and Health*, 35(6), 767-778. <https://doi.org/10.1007/s10653-013-9533-z>
- Hussain, B., Ashraf, M. N., Shafeeq-ur-Rahman, N., Abbas, A., Li, J., & Farooq, M. (2021). Cadmium stress in paddy fields: Effects of soil conditions and remediation strategies. *Science of the Total Environment*, 754, Article 142188. <https://doi.org/10.1016/j.scitotenv.2020.142188>
- Ionata, E., Caputo, E., Mandrich, L., & Marcolongo, L. (2024). Moving towards biofuels and high-value products through phytoremediation and biocatalytic processes. *Catalysts*, 14(2), Article 118. <https://doi.org/10.3390/catal14020118>
- Ji, W., Chen, Z., Li, D., & Ni, W. (2012). Identifying the criteria of cadmium pollution in paddy soils based on a field survey. *Energy Procedia*, 16, 27-31. <https://doi.org/10.1016/j.egypro.2012.01.006>
- Khan, A., Khan, M. S., Shafique, M. A., Khan, Q., & Saddiq, G. (2024). Assessment of potentially toxic and mineral elements in paddy soils and their uptake by rice (*Oryza sativa* L.) with associated health hazards in district Malakand, Pakistan. *Heliyon*, 10(7), Article e28043. <https://doi.org/10.1016/j.heliyon.2024.e28043>

- Li, X., Xiao, J., Salam, M. M. A., Ma, C., & Chen, G. (2020). Impacts of bamboo biochar on the phytoremediation potential of *Salix psammophila* grown in multi-metals contaminated soil. *International Journal of Phytoremediation*, 23(4), 387-399. <https://doi.org/10.1080/15226514.2020.1816893>
- Liu, Y., Ge, T., Van Groenigen, K. J., Yang, Y., Wang, P., Cheng, K., Zhu, Z., Wang, J., Li, Y., Guggenberger, G., Sardans, J., Penuelas, J., Wu, J., & Kuzyakov, Y. (2021). Rice paddy soils are a quantitatively important carbon store according to a global synthesis. *Communications Earth & Environment*, 2(1), Article 154. <https://doi.org/10.1038/s43247-021-00229-0>
- Mandal, J., Jain, V., Sengupta, S., Rahman, M. A., Bhattacharyya, K., Rahman, M. M., Golui, D., Wood, M. D., & Mondal, D. (2023). Determination of bioavailable arsenic threshold and validation of modelled permissible total arsenic in paddy soil using machine learning. *Journal of Environmental Quality*, 52(2), 315-327. <https://doi.org/10.1002/jeq2.20452>
- Nuruzzaman, M., Bahar, M. M., & Naidu, R. (2025). Diffuse soil pollution from agriculture: Impacts and remediation. *Science of the Total Environment*, 962, Article 178398. <https://doi.org/10.1016/j.scitotenv.2025.178398>
- Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., Shamseer, L., Tetzlaff, J. M., Akl, E. A., Brennan, S. E., Chou, R., Glanville, J., Grimshaw, J. M., Hróbjartsson, A., Lalu, M. M., Li, T., Loder, E. W., Mayo-Wilson, E., McDonald, S., McGuinness, L. A., Stewart, L. A., Thomas, J., Tricco, A.C., Welch, V. A., Whiting, P. and Moher, D. (2021). The PRISMA 2020 statement: An updated guideline for reporting systematic reviews. *BMJ*, 372, Article n71. <https://doi.org/10.1136/bmj.n71>
- Peng, H., Yang, X., Jiang, L., & He, Z. (2005). Copper phytoavailability and uptake by *Elsholtzia splendens* from contaminated soil as affected by soil amendments. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering*, 40(4), 839-856. <https://doi.org/10.1081/ESE-200048283>
- Rasin, P., Ashwathi, A. V., Basheer, S. M., Haribabu, J., Santibanez, J. F., Garrote, C. A., Arulraj, A., & Mangalaraja, R. V. (2025). Exposure to cadmium and its impacts on human health: A short review. *Journal of Hazardous Materials Advances*, 17, Article 100608. <https://doi.org/10.1016/j.hazadv.2025.100608>
- Sabir, M., Zia-Ur-Rehman, M., Hakeem, K. R., & Saifullah, N. (2015). Phytoremediation of metal-contaminated soils using organic amendments. In M. H. Fulekar, B. S. Rawat, & K. R. Hakeem (Eds.), *Soil remediation and plants: Prospects and challenges* (pp. 503-523). Academic Press. <https://doi.org/10.1016/B978-0-12-799937-1.00017-6>
- Shahbandeh, M. (2025). *World rice acreage 2010-2023*. Statista. <https://www.statista.com/statistics/271969/world-rice-acreage-since-2008/>
- Sharma, J. K., Kumar, N., Singh, N. P., & Santal, A. R. (2023). Phytoremediation technologies and their mechanism for removal of heavy metal from contaminated soil: An approach for a sustainable environment. *Frontiers in Plant Science*, 14, Article 1076876. <https://doi.org/10.3389/fpls.2023.1076876>
- Tamma, A. A., Lejcuś, K., Fiałkiewicz, W., & Marczak, D. (2025). Advancing phytoremediation: A review of soil amendments for heavy metal contamination management. *Sustainability*, 17(13), Article 5688. <https://doi.org/10.3390/su17135688>

- Tang, L., Hamid, Y., Zehra, A., Shohag, M. J. I., He, Z., & Yang, X. (2020). Endophytic inoculation coupled with soil amendment and foliar inhibitor ensure phytoremediation and agro-production in cadmium contaminated soil under oilseed rape-rice rotation system. *Science of the Total Environment*, 748, Article 142481. <https://doi.org/10.1016/j.scitotenv.2020.142481>
- Wang, H., Shi, C., Zhang, Q., Guo, D., Liu, S., & Yu, Q. (2024). Biochar assists phytoremediation of cadmium by regulation of rhizosphere microbiome in paddy fields. *Environmental Technology & Innovation*, 36, Article 103757. <https://doi.org/10.1016/j.eti.2024.103757>
- Wang, J., Deng, P., Wei, X., Zhang, X., Liu, J., Huang, Y., She, J., Liu, Y., Wan, Y., Hu, H., Zhong, W., & Chen, D. (2023). Hidden risks from potentially toxic metal(loid)s in paddy soils-rice and source apportionment using lead isotopes: A case study from China. *Science of the Total Environment*, 856, Article 158883. <https://doi.org/10.1016/j.scitotenv.2022.158883>
- Wang, Y., Li, S., Wang, X., Xu, J., Li, T., Zhu, J., Yang, R., Wang, J., Chang, M., & Wang, L. (2021). Biochelator assisted phytoremediation for cadmium (Cd) pollution in paddy field. *Sustainability*, 13(21), Article 12170. <https://doi.org/10.3390/su132112170>
- Wiszniewska, A., Hanus-Fajerska, E., Muszyńska, E., & Ciarkowska, K. (2016). Natural organic amendments for improved phytoremediation of polluted soils: A review of recent progress. *Pedosphere*, 26(1), 1-12. [https://doi.org/10.1016/S1002-0160\(15\)60017-0](https://doi.org/10.1016/S1002-0160(15)60017-0)
- Witt, C., & Haeefe, S. (2005). Paddy soils. In D. Hillel (Ed.), *Encyclopedia of soils in the environment* (Vol. 3, pp. 141-150). Academic Press. <https://doi.org/10.1016/B0-12-348530-4/00286-1>
- Yang, X., Peng, H., Jiang, L., & He, Z. (2005). Phytoextraction of copper from contaminated soil by *Elsholtzia splendens* as affected by EDTA, citric acid, and compost. *International Journal of Phytoremediation*, 7(1), 69-83. <https://doi.org/10.1080/16226510590915855>
- Zhao, S., Ye, X., Chen, D., Zhang, Q., Xiao, W., Wu, S., Hu, J., Gao, N., & Huang, M. (2022). Multi-component passivators regulate heavy metal accumulation in paddy soil and rice: A three-site field experiment in South China. *Toxics*, 10(5), Article 259. <https://doi.org/10.3390/toxics10050259>
- Zheng, C., Wang, X., Liu, J., Ji, X., & Huang, B. (2019). Biochar-assisted phytoextraction of arsenic in soil using *Pteris vittata* L. *Environmental Science and Pollution Research*, 26(36), 36688-36697. <https://doi.org/10.1007/s11356-019-06688-5>
- Zhou, T., Wu, L., Christie, P., Luo, Y., & Fornara, D. A. (2018). The efficiency of Cd phytoextraction by *S. plumbizincicola* increased with the addition of rice straw to polluted soils: The role of particulate organic matter. *Plant and Soil*, 429, 321-333. <https://doi.org/10.1007/s11104-018-3698-2>
- Zhou, X., Lei, B., Yin, D., Kang, J., He, Z., He, T., & Xu, X. (2023). Application potential of biofertilizer-assisted *Pennisetum giganteum* in safe utilisation of mercury-contaminated paddy fields. *Journal of Environmental Management*, 348, Article 119291. <https://doi.org/10.1016/j.jenvman.2023.119291>

Callus and Embryo Induction from Anther Culture of *Cucumis melo* L. cv. Glamour

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ABSTRACT

To establish an efficient haploid production system for melon breeding, this study was conducted at the Laboratory of Universiti Malaysia Kelantan, Kelantan, Malaysia, in 2023, to evaluate the effects of cold pretreatment, surface sterilisation duration, and plant growth hormone concentrations on callus induction and embryogenesis in anther culture of *Cucumis melo* cv. Glamour, an important commercial melon cultivar. Early morning, unpollinated male flowers were harvested from 35

to 45-day-old plants. Sodium hypochlorite (NaOCl) containing Tween-20 was used as a surface sterilisation solution for anthers at 4, 7, and 4°C after cold pretreatment for 10, 20, and 30 minutes. Cultures were established on Murashige and Skoog (MS) basal medium supplemented with varying concentrations of 2, 4-D and BAP. Incubation was carried out at 25±2 °C under dark conditions. A ruler and graph paper were used to measure average callus size. Embryogenic structures were morphologically screened and validated through flow cytometry. All treatments resulted in 100% callus induction, but callus size and quality varied with treatment

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conditions. The 3-day cold pretreatment significantly enhanced callus formation, while 20-minute sterilisation was optimal for healthy, viable callus. The medium containing 2, 4-D (5.0 mg/L) and BAP (0.5 mg/L) produced the largest callus and highest proliferation rate, whereas BAP (4.0 mg/L) combined with NAA (0.05 mg/L) was most effective for inducing embryogenesis. A flow cytometry analysis revealed that most calli were mixoploid. The results contribute to the development of a new protocol for anther culture in *C. melo*, which may be useful in improving the melon crop.

Keywords: Anther culture, callus, embryo, haploid, plant growth regulators

INTRODUCTION

Plant tissue culture is a foundational tool in modern plant biotechnology, enabling the regeneration of whole plants from isolated cells or tissues under controlled and sterile conditions. The concept of cellular totipotency, first proposed by Gottlieb Haberlandt in 1902 and later validated by Knudson's successful in vitro germination of orchid seeds in 1922, laid the foundation for what is now an essential technique in plant propagation and breeding (Bridgen et al., 2018; Thorpe, 2007). Among various in vitro approaches, androgenesis (anther culture) and gynogenesis (ovary culture) have emerged as powerful strategies for producing haploid and doubled haploid (DH) lines, which significantly accelerate the development of homozygous lines for hybrid seed production.

Tissue culture success relies on multiple factors, including the type of explant, culture medium composition, hormone balance, and pre-treatments applied to enhance response. The MS medium is popular for being a full supplement medium; however, it usually needs optimisation to meet the species requirements (Phillips & Garda, 2019). Hormones involved in the manipulation of in vitro morphogenesis, particularly in the induction of callus and somatic embryogenesis and shoot regeneration effects, are the plant growth regulators (2, 4-D and NAA as auxins and BAP and TDZ for cytokinins). Other medium constituents, such as sucrose, vitamins, and jellifying agents, also promote the in vitro plant growth.

Anther culture has been successfully applied in several cucurbit species, such as cucumber (*Cucumis sativus*), squash (*Cucurbita pepo*) and bitter melon (*Momordica charantia*) to induce callus and haploids (Asadi et al., 2018; Habiba, 2016; Nguyen et al., 2019). Nevertheless, species and cultivar-specific responses are well known, and protocols often need to be fine-tuned. Likewise, ovary culture is an effective alternative for haploid production, although they are less efficient than anther culture, which is useful in situation for anther culture has very low efficiency or is impossible (Deng et al., 2020; Ozsan et al., 2017). Especially, the fertility of both methods predominates upon pretreatments like cold and heat shocks affecting microspore reprogramming and embryogenesis initiation (Kurtar et al., 2016; Rakha et al., 2012).

Although the in vitro culture of the plant is well established, *C. melo* cv. Glamour has not yet been fully exploited for the stable protocols of haploid and double haploid plants production. Even though both androgenesis and gynogenesis have been investigated in other cucurbits, there are no optimised and reliable procedures established in summer squash (SS), hampering its use in breeding programs that need homozygous lines as parents. Furthermore, although cold preconditioning is widely used to increase the induction of callus, its impact on the reproductive tissues of *C. melo* cv. Glamour has not been completely explained. However, its role in anther culture is still unclear and deserves investigation. Some studies found that the cold treatment could suppress gynogenesis in the cucurbits; hence, the conflicting reports regarding the impact of cold exposure on anthers remain highly controversial (Ibrahim et al., 2023a).

Furthermore, the hormone-mediated impact on callus and embryo formation was not investigated systematically in this cultivar. Overall, most comparative studies looked at select numbers of hormone combinations, usually with no statistical resolution. Understanding how auxins and cytokinins interact across a wider range of concentrations is essential for controlling regeneration potential and embryogenic response. Finally, although tissue culture-induced ploidy variations, including mixoploidy and polyploidy, are well documented, there have been only a limited number of reports describing the use of flow cytometric examination of genetic stability in *C. melo* regenerants or clones obtained from anther or ovary cultures.

This study aimed to address these gaps by examining the effects of cold pretreatment, sterilisation duration, and hormone concentration on the induction of callus and embryo in anther culture of *C. melo* cv. Glamour. In addition to morphological evaluation, flow cytometry was employed to assess ploidy level, thereby contributing to the development of a robust in vitro protocol for future melon breeding and genetic improvement efforts.

MATERIALS AND METHODS

Anther Culture

The experiments were conducted in 2023 at the Laboratory of Universiti Malaysia Kelantan, Kelantan, Malaysia. Male flower buds of *C. melo* were collected from 35 to 45-day-old plants early in the morning when the temperature was low, and the plant water content was high, as suggested by Hatfield and Prueger (2015). Buds were pre-treated at 4-7 °C for 0, 1, 2, or 3 days. Surface sterilisation involved immersion in 70% ethanol for one minute, rinsing with autoclaved distilled water, shaking in a commercial stock solution of 5.25% sodium hypochlorite (NaOCl) supplemented with Tween-20 for 10, 20, or 30 minutes, followed by three final rinses with autoclaved distilled water. Sterilised flower buds were placed on filter paper to remove residual sterilant and water.

Anthers were excised from filaments and petals, then separated into segments and cultured on MS medium supplemented with plant growth regulators (Figure 1). Cultures were maintained at 25 ± 2 °C under dark conditions for callus induction and embryo initiation. Ploidy level of the calli was assessed using flow cytometry.

To prepare one litre of culture medium, 4.33 g MS basal powder, 30 g sucrose, 5.5 g agar, and 0-5.0 mg/L of plant growth regulators were used. The pH was adjusted to 5.6-5.8 to provide optimal conditions for explant growth. Autoclaving was conducted at 121 °C for 15 minutes as a sterilisation step (Ahmed et al., 2016 & Ibrahim et al., 2023b).

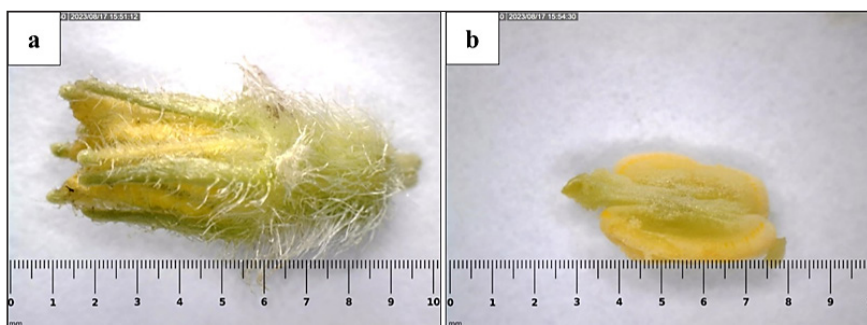


Figure 1. The condition and approximate size of (a) a closed *C. melo* male flower and (b) anther used in this study

Flow Cytometry

To determine the samples' ploidy levels using flow cytometry, each sample was chopped into small pieces and placed in separate petri dishes. Nuclei were extracted by manually chopping the tissues in lysis buffer. Among the three tested buffers (LBO1, Tris-MgCl₂, and Otto), LBO1 was chosen as it gave the clearest histogram peaks as suggested by Midin et al. (2017) and Tomaszewska et al. (2021).

The nuclei suspensions were filtered through a 50 µm nylon mesh, then treated with RNase A and propidium iodide (PI). PI was used as it binds to DNA and produces accurate fluorescence peaks with low variation (Doležal and Bartos, 2005; Doležal et al., 2007; Midin et al., 2017).

Samples were incubated at 4 °C for 5 minutes before analysis through a FACSCalibur flow cytometer (Becton Dickinson, USA) with a 488 nm laser. Each run recorded 5000 events across 1024 channels. Young leaves or cotyledons from non-tissue culture plants were used as ploidy controls. Peak data were analysed using CellQuest3Q software, following the method by Midin et al. (2017).

Experimental Design and Statistical Analyses

All the experiments were arranged in a Completely Randomised Design (CRD) with five replications per treatment. The one-way ANOVA was used to analyse the statistical

data of cold-pre-treated durations, surface sterilisation exposure time and plant growth regulator combinations. Data was analysed and segregated using Duncan Multiple Range Test (DMRT) at a significance level of $P \leq 0.05$. SPSS software (Version 25.0) was used for the statistical analyses.

RESULTS

Preliminary Study

In the preliminary study, anther culture successfully induced callus formation under a 0-day cold pretreatment and 10-minute 5.25% NaOCl + 3 drops of Tween-20 surface sterilisation, following the methods by Song et al. (2007) and Golabadi et al. (2017). Among the hormone combinations tested, 2, 4-D (1.5 mg/L) and BAP (0.75 mg/L) produced the highest amount of callus and the largest callus size. Therefore, these three conditions, which are cold pretreatment duration, sterilisation time, and hormonal concentrations, were selected for further optimisation. The use of 2, 4-D and BAP in the induction medium was followed by Kurtar et al. (2016).

The Effect of Cold Pretreatment

Anther-derived callus induction and proliferation were periodically examined during the cold pretreatment, and the callused structures were evaluated at eight to twelve weeks of culture. Surface sterilisation was standardised at 10 minutes of 5.25% NaOCl along with Tween-20 treatment, and a hormonal regime of 2, 4-D (1.5 mg/L) + BAP (0.75 mg/L) was uniform in all treatments. In the current study, formation of indicative callus was observed in all treatment groups irrespective of duration of cold pretreatment (0, 1, 2, 3 days at 4-7 °C) or surface sterilisation time period (10, 20, 30 minutes in 5.25% NaOCl along with Tween-20) and each treatment was found with 100% callus induction as recognized in some research (Ahmed et al., 2015). However, no callus was observed in hormone-free MS0 media, indicating that callus formation was strictly dependent on the presence of plant growth hormones. Induction rates were calculated by dividing the number of replicates producing calli by the total number of replicates and expressing the result as a percentage (Naïtchédé et al., 2023).

As shown in Figure 2 (2A-2D) and Table 1, all cold pretreatment durations were capable of triggering callus induction in less than four weeks. Although 100% induction was recorded across all treatments, the proliferation and the callus size varied. The largest callus was observed in media 2D (3-day cold pretreatment), followed by media 2B, 2C, and 2A. A one-way ANOVA was conducted to statistically assess whether the mean callus sizes differed significantly among the cold pretreatment groups. The analysis yielded $F(3, 16) = 3.297$, $p = 0.05$, indicating that differences among group means were not statistically significant at the conventional level, thus supporting the null hypothesis.

However, post hoc analysis revealed significant pairwise differences between certain treatment groups. This highlights that while overall group variation was not statistically confirmed, specific comparisons reveal meaningful differences. In Table 1, treatment means followed by different letters denote statistically significant differences.

The Effect of Surface Sterilisation (Exposure Time)

In assessing the effect of surface sterilisation duration on callus induction and proliferation, anther cultures were monitored periodically, and results were also analysed after eight to twelve weeks of culture. Based on preliminary callus induction results, cold pretreatment was fixed at 0-day duration, and the hormonal combination of 2, 4-D + BAP was maintained across all

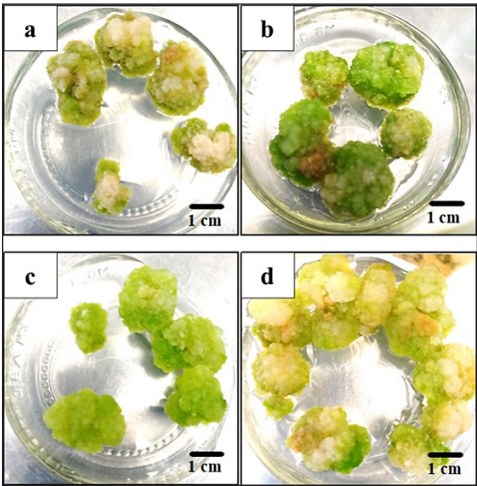


Figure 2. Callus proliferation after 12 weeks, under different durations of cold pretreatments (CPT), (a): 0-day CPT, (b): 1-day CPT, (c): 2-day CPT, and (d): 3-day CPT

Table 1
The effect of different durations of cold pretreatment on callus induction and proliferation

Experiment	Media (Label)	Duration of Cold pretreatment (4 -7 °C)	Percentage of Callus Induction	Average Diameter of the Callus (mm) after 12 Weeks (5 Replicates)
Assessment of the duration of cold pretreatment	Figure 2a	0 day	100%	16.2 ± 0.97 ^b
	Figure 2b	1 day	100%	18.2 ± 1.86 ^b
	Figure 2c	2 days	100%	16.8 ± 1.39 ^{ab}
	Figure 2d	3 days	100%	22.4 ± 1.78 ^a
MS0	-	0 day	0%	-

Note. *Means with the same letter were not significantly different from each other (P<0.05 ANOVA followed by DMRT)

treatments. All surface sterilisation durations (10, 20, and 30 minutes of NaOCl + Tween-20) resulted in 100% callus induction (Table 2). A 100% induction rate was recorded when all replicates within a treatment successfully formed calli.

Callus induction was found to be strongly dependent on the presence of plant growth regulators. No callus formed in the hormone-free medium (MS0), regardless of sterilisation duration, emphasising that hormonal supplementation is critical for initiating callogenesis. While surface sterilisation duration was a controlled variable, its influence on callus formation was only evident in the presence of hormones. Positive callus induction was observed in anthers sterilised for 10, 20, and 30 minutes using NaOCl + Tween-20 in 2E-2G media (Figure 3). All treatments successfully induced calli, with variations in proliferation and callus size depending on the sterilisation duration.

A one-way ANOVA was conducted to compare the effects of different surface sterilisation durations (5.25% NaOCl + Tween-20) on callus size (mm). The analysis indicated no statistically significant differences among the groups $F(2, 12) = 3.764$, $p = 0.054$). The p -value of 0.054 slightly exceeds the 0.05 threshold, indicating that the differences in callus size across sterilisation durations were not statistically significant

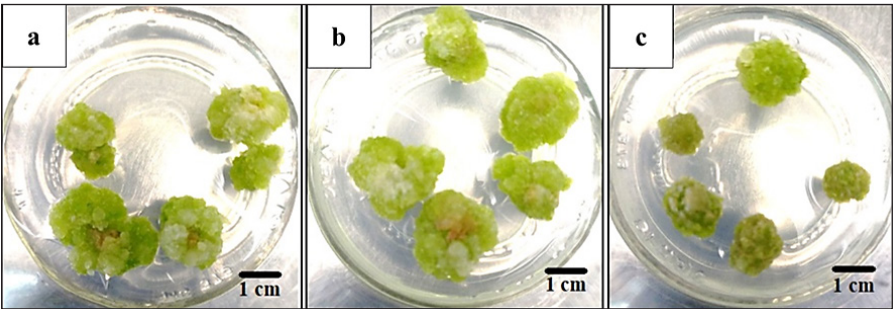


Figure 3. Callus proliferation after 12 weeks, under different durations of surface sterilisation (5.25% NaOCl + 3 drops of Tween-20), (a) 10-minute, (b) 20-minute, and (c) 30-minute

Table 2
The effect of different durations of surface sterilisation on callus induction and proliferation

Experiment	Media (Label)	Duration of surface sterilisation (10-30 min 5% NaOCl + Tween-20)	Percentage of Callus Induction	Average Diameter of the Callus (mm) after 12 Weeks (5 Replicates)
Assessment of the duration of surface sterilisation	Figure 3a	10 min	100%	15.00 ± 1.76 ^{ab}
	Figure 3b	20 min	100%	16.80 ± 0.37 ^a
	Figure 3c	30 min	100%	12.40 ± 0.81 ^b
MS0	-	-	0%	-

Note. *Means with the same letter were not significantly different from each other (P<0.05 ANOVA followed by DMRT)

at the 5% level, thus supporting the null hypothesis of equal means. However, post hoc analysis revealed significant differences between specific pairs of groups, suggesting that pairwise differences existed despite the overall ANOVA result.

The Effect of Hormone Concentrations and Combinations

In evaluating hormonal concentrations and combinations for *C. melo* anther-derived callus induction and proliferation, eight treatments containing different concentrations of 2, 4-D and BAP were assessed after four weeks. All treatments used a consistent cold pretreatment (0-day) and sterilisation duration (10 min NaOCl + Tween-20). The hormone concentrations were adapted from protocols by Nguyen et al. (2019) and Kurtar et al. (2016). Successful callus induction and proliferation (100%) was observed in most treatments (4d-4h), except for several treatments (4a, 4b, and 4c), which showed no response (Figure 4). In treatments with positive induction, green and healthy calli were observed, with size variation depending on the concentrations of 2, 4-D and BAP (Table 3).

Callus growth was assessed by measuring the area using graph paper under a dissection microscope. Media in Figures 4b and 4c showed no induction, with average callus sizes of $1.30 \pm 0.12 \text{ mm}^2$ and $0.95 \pm 0.17 \text{ mm}^2$, respectively. Both were smaller than the average anther size prior to culture ($1.55 \pm 0.55 \text{ mm}^2$), indicating necrosis. These media contained only high concentrations of 2, 4-D, suggesting that 2, 4-D alone at 2.0-5.0 mg/L was not conducive to anther-derived callus induction in *C. melo*.

The rate of callus proliferation was calculated as a relative change, where the absolute value of the change was divided by the mean of the values and multiplied by 100% (Brauen et al., 2021).

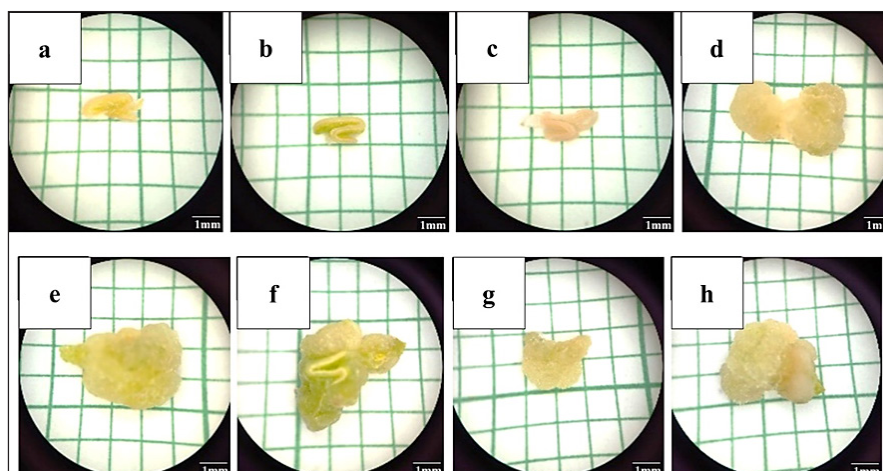


Figure 4. Callus proliferation after four weeks, under different hormonal concentrations and combinations, (a) MS0, (b) 2, 4-D (2.0 mg/L), (c) 2, 4-D (5.0 mg/L), (d) 2, 4-D (2.0 mg/L) + BAP (0.5 mg/L), (e) 2, 4-D (2.0 mg/L) + BAP (1.0 mg/L), (f) 2, 4-D (5.0 mg/L) + BAP (0.5 mg/L), (g) 2, 4-D (5.0 mg/L) + BAP (1.0 mg/L), and (h) 2, 4-D (1.5 mg/L) + BAP (0.75 mg/L)

Table 3
The effect of different durations of surface sterilisation on callus induction and proliferation

Experiment	Media (Label)	Hormonal Combination	Percentage of Callus Induction	Average Size of the Callus (mm ²) (5 Replicates)	Rate of Proliferation
Assessment of hormonal combination	Figure 4a	MS0	0%	Necrosis	0.00 ± 0.00 ^c
	Figure 4b	2, 4-D (2.0 mg/L) + BAP (0 mg/L)	0%	Necrosis	0.00 ± 0.00 ^c
	Figure 4c	2, 4-D (5.0 mg/L) + BAP (0 mg/L)	0%	Necrosis	0.00 ± 0.00 ^c
	Figure 4d	2, 4-D (2.0 mg/L) + BAP (0.5 mg/L)	100%	4.95 ± 0.86 ^{ab}	2.10 ± 0.50 ^b
	Figure 4e	2, 4-D (2.0 mg/L) + BAP (1.0 mg/L)	100%	4.50 ± 1.02 ^{bc}	1.90 ± 0.66 ^{bc}
	Figure 4f	2, 4-D (5.0 mg/L) + BAP (0.5 mg/L)	100%	6.45 ± 0.34 ^a	4.62 ± 1.3 ^{6a}
	Figure 4g	2, 4-D (5.0 mg/L) + BAP (1.0 mg/L)	100%	3.10 ± 0.23 ^{cd}	1.00 ± 0.15 ^{bc}
Positive control	Figure 4h	2, 4-D (1.5 mg/L) + BAP (0.75 mg/L)	100%	4.20 ± 0.82 ^{bc}	1.71 ± 0.53 ^{bc}

One-way ANOVA followed by Duncan’s Multiple-Range Test (DMRT) was used to evaluate the significance of differences between treatments (Table 4). Among all treatments, media in Figure 4f produced the largest callus area (6.45 ± 0.34 mm²) and the highest proliferation rate (4.62 ± 1.36), both significantly greater (p < 0.05) than other treatments. Media in Figure 4d (4.95 ± 0.86 mm²) also yielded significantly larger callus sizes compared to the rest. Conversely, media in Figures 4b and 4c exhibited minimal growth and necrosis, with no proliferation rate recorded.

Statistical analysis confirmed that the combination of 5.0 mg/L 2, 4-D and 0.5 mg/L BAP (Figure 4f) was the most effective treatment overall. Media 4d and 4e also showed significant performance, with relatively large callus areas (4.95 ± 0.86 mm² and 4.50 ± 1.02 mm², respectively). A significant correlation between media composition and callus size emphasises the critical role of hormonal balance in supporting callus induction and proliferation in *C. melo*. In contrast, treatments in Figures 4a-4c were found unsuitable, likely due to necrosis resulting from hormonal imbalance or absence.

Embryogenic Callus Initiation from *C. melo* Anther Culture

The optimised culture conditions (3 days cold pretreatment, surface sterilisation with a 20 min 5.25% NaOCl + 3 drops of Tween-20 wash and a 5.0 mg/L 2, 4-D + 0.5 mg/L BAP hormonal treatment) were then used to culture fresh anthers to achieve maximum calli

induction. The developed calli were transferred regularly in a fresh medium in order to induce maturation, in each sub-culturing plan, the same sub-culturing method was used as that of sub-culturing based on Asadi et al. (2018), reporting that calli sub-cultured every 21 days remarkably enhanced growth of callus and morphogenic competence in cucumber (*Cucumis sativus*).

The callus of the maturation culture was sub-cultured to fresh medium supplemented with NAA (0.05 mg/L) + BAP (4.0 mg/L) after 12 weeks of incubation, as described by Kurtar et al. (2016) previously, for embryogenic callus initiation. The objective of this experiment was to induce embryogenic callus or shoots. The concentration of cytokinin BAP was raised in the embryogenic callus initiation over the callus induction and maturation medium. Cytokinin was applied at a higher rate than auxin, resulting in an 80:1 cytokinin-auxin ratio (4.0 mg/L BAP versus 0.05 mg/L NAA). It was decided to use this specific ratio since intermediate hormone ratios promote unorganised callus growth, while an extreme shift toward high levels of cytokinins and minimal auxin concentrations is crucial for suppressing auxins' inhibitory effects on somatic embryogenesis, resulting in the differentiation of embryogenic cells and embryo-like structures (ELS) in *Cucumis* species (Ikeuchi et al., 2013; Kurtar et al., 2016).

In twelve weeks, the observation on the progress of achieving embryogenic callus on medium 5a (0.05 mg/L NAA + 4.0 mg/L BAP) showed that the callus survived but in a slow growth pattern. The calli were selected randomly and divided into five different media sets. Each set was treated with 5a-5e media separately (Figure 5). Medium 5a, which maintained the hormonal combination of NAA (0.05 mg/L) + BAP (4.0 mg/L), was used as a positive control for the embryogenic callus initiation. After four weeks, the difference between the growth rate of callus in each medium was observed. Notably, the highest formation of embryogenic callus was observed in medium 5b (BAP 4.0 mg/L), suggesting that BAP alone, at a high concentration, was more effective than combinations with NAA.

Medium 5b showed the best response by the highest formation (87%) of embryogenic callus or embryo-like structures (ELS) with globular and pre-globular shaped callus, followed by media 5a, 5c, 5d, and 5e (Figure 5). Embryogenic callus initiation culture results were summarised in Table 4. The embryogenic callus formation percentage was calculated by the number of calli which gave rise to embryogenic calli over the total number of calli which were transferred to embryo maturation media and expressed as a percentage (%) (Naïtchédé et al., 2023). The features of embryogenic and non-embryogenic were listed in Table 5. The association between unique hormonal combinations and embryogenic callus percentage were determined by the Chi-Square Test of Independence. The extremely low p-value of .001 (X^2 (4, N = 260) = 18.056, p = .001) suggests that media selection has a significant impact on discrimination between embryogenic and non-embryogenic callus.

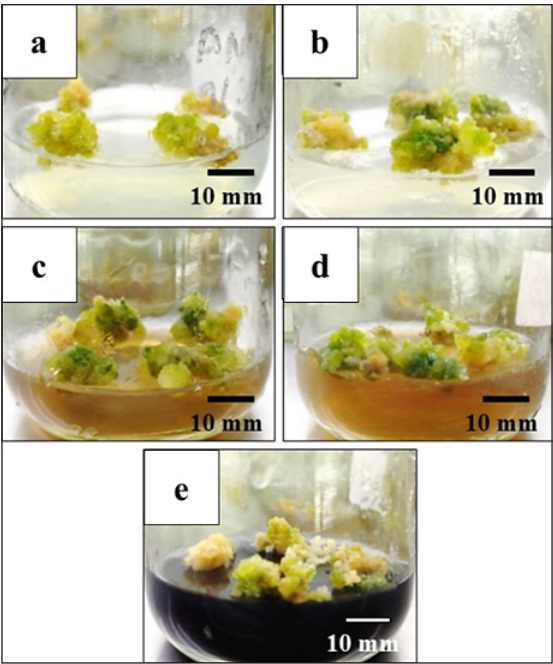
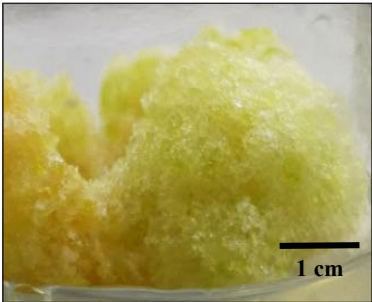


Figure 5. Embryogenic callus growth on initiation media, (a) NAA (0.05 mg/L) + BAP (4.0 mg/L), (b) BAP (4.0 mg/L), (c) NAA (0.05 mg/L) + BAP (4.0 mg/L) + AgNO₃ (10 mg/L) + ascorbic acid (0.5 mg/L), (d) BAP (4.0 mg/L)+ AgNO₃ (10 mg/L) + ascorbic acid (0.5 mg/L), and (e) BAP (4.0 mg/L) + AgNO₃ (10 mg/L) + ascorbic acid (0.5 mg/L) + activated charcoal (2.5 g/l)

Table 4
Embryogenic callus initiation from *C. melo* anther culture in five different media

Experiment	Media (Label)	Hormonal Combination	Percentage of Embryogenic Callus Formation	Percentage of Non-Embryogenic Callus Formation
Embryogenic callus initiation (52 replicates)	Figure 5a	NAA (0.05 mg/L) + BAP (4.0 mg/L)	75%	25%
	Figure 5b	NAA (0 mg/L) + BAP (4.0 mg/L)	87%	14%
	Figure 5c	NAA (0.05 mg/L) + BAP (4.0 mg/L) + AgNO ₃ (10 mg/L) + Ascorbic Acid (0.5 mg/L)	73%	27%
	Figure 5d	NAA (0 mg/L) + BAP (4.0 mg/L) + AgNO ₃ (10 mg/L) + Ascorbic Acid (0.5 mg/L)	60%	40%
	Figure 5e	NAA (0 mg/L) + BAP (4.0 mg/L) + AgNO ₃ (10 mg/L) + Ascorbic Acid (0.5 mg/L) + 2.5 g Activated Charcoal	52%	48%
	-	MS0	0%	100%

Table 5
Characteristics of embryogenic and non-embryogenic callus from anther culture of *C. melo*

Embryogenic callus	Characteristics	Non-embryogenic callus
		
Pre-globular/ globular-shaped	Morphology	Irregular-shaped
Compact	Structure	Loose
Not friable	Friability	Easily crumbles, friable
Green or yellowish-green	Colour	Light green or yellowish green
Opaque	Transparency	Semi-transparent

DISCUSSION

Cold pretreatment is a commonly applied technique in plant tissue culture, often used to enhance callus or embryogenic responses. In this study, its role in *C. melo* anther-derived callus formation was explored and found to align with findings in other species, such as *Capsicum annuum* L. (Haridhi, 2023), where pretreatment promoted callus initiation. Concerning *C. melo*, even negative effects of cold pretreatment on ovary-derived calli induction and proliferation have been previously documented (Metwally et al., 1998; Rakha et al., 2012). This study has presented the advanced anther culture technique to show that cold application is beneficial for anther cultures, probably through its various effects on physiological processes, such as the delayed senescence of anther walls, promotion of symmetric divisions of microspores, and release of necessary substances (amino acids and heat-shock proteins), as reported before (Touraev et al.). Similarly, cold stress can induce the shift of microspore development from the gametophytic to sporophytic pathways, leading to the formation of callus or embryogenesis (Mishra & Rao, 2013). According to this study, a pre-cold pretreatment for 3 days was certainly recommendable for *C. melo* anthers, due to the fact that it can result in the maximum callus size in the proliferation stage. These results reveal and confirm that the better treatment for promoting callus growth is a 3-day cold pretreatment than the other cold treatments.

Sterilisation treatments and cold pretreatments are also important factors in the success of anther culture, in addition to cold pretreatment. Various factors, such as the physiological status of the donor plants, the size, age and type of the explant, and the concentration and

duration of a sterilising agent, affect the efficiency of sterilisation. Hence, it is impossible to design a single Objective protocol of disinfection, and often the optimal series of operations may have Species-specific optimal disinfection and get the highest number of viable and aseptic Acta explants (Ahmadpoor et al., 2022). Sodium hypochlorite or NaOCl is considered a standard sterilising agent, due to its strong antibacterial and proteolytic abilities. Its active ingredient is chlorine, which results in an irreversible oxidation of bacterial enzymes, leading to a disruption of the metabolic process. Nevertheless, its phytotoxicity is also well reported (Mavani et al., 2020). While higher concentrations of NaOCl can enhance sterilisation levels, it also elevates the possibility of tissue damage and cytotoxic effects (Fitri et al., 2020; Mahood et al., 2022). Therefore, the concentration and exposure duration must be optimised based on the tissue type, in which lower concentrations are used for delicate tissues, and stronger treatments are applied for more robust explants like seeds (Bridgen et al., 2018). Previous studies reported NaOCl treatment durations ranging from 4 to 20 minutes for Cucurbitaceae species (Nguyen et al., 2019; Rakha et al., 2012). Lower NaOCl concentrations were typically applied for extended durations to enhance the aseptic rate.

In this study, 30 minutes of 5.25% NaOCl treatment was considered relatively long, yet the enclosed anther within the flower bud tolerated the exposure and produced healthy calli. This may be due to the protective role of the outer floral parts (petals and sepals), which buffered the anther from direct chemical stress. In another study, certain tissues in Cucurbitaceae species are quite robust and not even affected by this treatment. For example, Kurtar et al. (2016) reported successful callus formation from anthers of *C. maxima* and *C. moschata*, although after the sepal removal during surface sterilisation.

The findings of this study confirmed the potential of 10, 20, and 30 min NaOCl + Tween-20 treatments to sterilise *C. melo* flower buds and aid the induction of anther-derived *C. melo* callus. Ten minutes was sufficient for sterilisation, and the treatment at 20 minutes (2F) led to the highest proliferating rate and the largest callus diameter (16.8 ± 0.374 mm), indicating the best conditions for callus growth. However, 30 min treatment (2G) induced a significantly smaller callus size (12.4 ± 0.812 mm). This observation suggests that the increased duration of NaOCl application has led to high cytotoxicity, thereby reducing the callus potential for further growth. Cytological and genotoxic effects of NaOCl in onion (*Allium cepa*) and garden pea (*Pisum sativum*) have been reported. It is able to interfere with the cell cycle primarily by inducing G2 arrest and inhibiting DNA synthesis, resulting in a blockage of cell development (Salazar-Mercado et al., 2019). According to this study, 20 minutes of surface sterilisation with 5.25% NaOCl + Tween-20 is desirable for *C. melo* anther cultures since it resulted in the highest callus size when they were in the proliferation stage. The treatment response rate: a moderate power but satisfactory for a trade-off between the sterilisation effect and the tissue viability.

In addition to cold pretreatment and surface sterilisation, hormonal types and concentrations in the culture medium are critical for callus induction. The observation that media containing only high levels of 2, 4-D failed to induce callus implies that 2, 4-D alone at 2.0-5.0 mg/L was not suitable for anther-derived callus induction in *C. melo*. In contrast, Kurtar et al. (2016) found that comparable concentrations of 2, 4-D successfully induced callusing in winter squash (*Cucurbita maxima* L.) and pumpkin (*Cucurbita moschata* Duch.) Since both species are members of the Cucurbitaceae family, these contrasting results demonstrate species-specific hormonal needs. This inconsistency highlights that although 2, 4-D at 2.0-5.0 mg/L was effective for winter squash and pumpkin, it was ineffective for melon (*C. melo*) when applied without cytokinin.

2, 4-D is among the most widely used synthetic auxins for promoting plant growth. It has been shown that auxin, in combination with other plant hormones, is essential for cell division and directional cell differentiation, impacting overall plant development (Simon & Petrášek, 2011). However, an imbalance, such as an excess of auxins relative to cytokinins, can inhibit proper callus formation. Typically, an intermediate ratio between auxins and cytokinins is optimal for callus induction (Ikeuchi et al., 2013). This is supported by the high callus induction observed in treatments combining 2, 4-D with BAP (4d-4o), and aligns with Skoog and Miller's (1957) principle that an intermediate auxin-cytokinin ratio promotes callus development in plant tissues (Ikeuchi et al., 2013). Moreover, the 0% induction in 4a medium (without hormones) is also consistent with the Kurtar et al. (2016) results.

It has been noted with some past reviews that although low concentrations of 2, 4-D, and other synthetic auxins can promote growth of the plant, the hormone is shown to stimulate abnormal plant growth at high concentrations (Song, 2014). In addition, 2, 4-D can induce epigenetic and genetic changes, such as DNA methylation and mutation. In somatic embryogenesis, high levels of 2, 4-D have been shown to suppress typical embryo development by affecting genetic and physiological processes at the cell level (García et al., 2019). These findings reinforce the importance of species-specific optimisation of hormone concentrations, particularly 2, 4-D, to support successful callus induction and proliferation, as demonstrated in this study and the work of Kurtar et al. (2016).

Further investigation focused on identifying conditions that specifically promote embryogenic callus formation. In this context, the observation from this study slightly differs from Ikeuchi et al. (2013) and Kurtar et al. (2016), who reported that a higher ratio of cytokinin-to-auxin promotes embryo formation or shoot regeneration. In this study, the absence of NAA, an auxin, in media 5b may have reduced the inhibitory effects on embryogenesis typically associated with auxins, thus favouring the formation of embryogenic callus.

In media 5c-5e, the addition of AgNO₃, ascorbic acid, and activated charcoal aimed to reduce the phenolic exudation and to enhance the callus growth (Demirkaya

& Çömlekçioğlu, 2021). Dangi et al. (2014) stated in their study that the addition of antioxidants such as polyvinylpyrrolidone (PVP), ascorbic acid, and citric acid has demonstrated a beneficial effect on explant survival. Nevertheless, the overall response of these treatments in this study showed that these supplements (AgNO₃, ascorbic acid and activated charcoal) were not significantly effective in promoting embryogenic callus formation as compared to media 5b, which might imply that these additives are good for eliminating the phenolic stress but had less nutritional impact on the embryogenic calli, or perhaps their concentrations might not be well adjusted. Demirkaya and Çömlekçioğlu (2021) also found such tendencies in other species, suggesting that the use of these additives could be largely context dependent.

Chi-Square analysis reveals that there is a significant influence of culture medium, hormonal combinations and additives on embryogenic callus induction. BAP (4.0 mg/L) was the best elicitor for the induction, while silver nitrate, ascorbic acid and activated charcoal showed a negative effect on embryogenic callus induction, indicating the need for optimisation of these compounds in a tissue culture regime. Further studies are required in order to understand the exact nature of these effects, particularly the balance of auxin and cytokinin and the role of additives such as AgNO₃ and activated charcoal.

To characterise the obtained calli, in particular embryogenic ones, the ploidy of the calli was measured by flow cytometry. The ploidy status of the anther-derived calli samples analysed deviated from expectation. Even though calli were considered to be haploid, flow cytometry revealed that they included both mixoploid and polyploid cells (Figure 6). As the calli were the object of the present investigation, chromosomal variation was more apparent than that of the adventitious shoots as reported by Krishna et al. (2016).

The reasons for the remarkable levels of unexpected ploidy observed in this study are numerous. Mitotic abnormalities, in particular failed cytokinesis, are probably among the factors contributing to this phenomenon. These errors can lead to unbalanced chromosomal content (aneuploidy or polyploidy) (Muenchrath et al., 2016; Potapova and Gorbis, 2016).

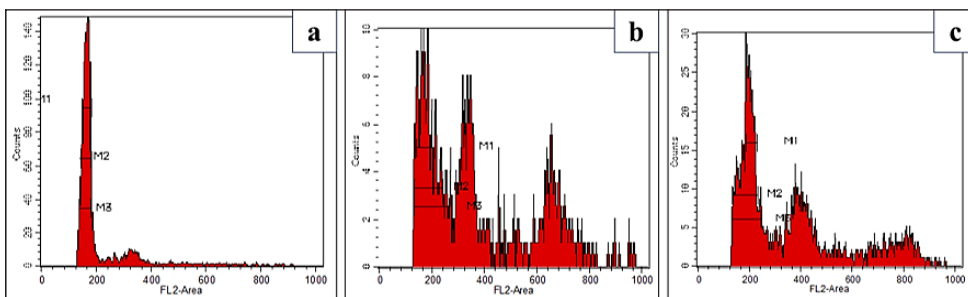


Figure 6. Histograms from Flow Cytometry analysis, (a) cotyledon as diploid external standard, (b) and (c) calli samples from anther culture

2017), which is the primary manifestation observed post analysis. Flow cytometry is also less sensitive for detecting aneuploidy, but aneuploidy can also occur. So, in order to obtain effective detection results, it is recommended to use flow cytometry combined with karyotyping (Eng et al., 2021). Chromosomes which fail to separate correctly due to errors in the mitotic machinery can cause arrest of the cell cycle at metaphase. At re-entry into mitosis, these cells may duplicate their chromosomes to generate polyploid cells (Eng et al., 2021; Zhou et al., 2017).

Polyploidization could also be associated with TCIV (Bairu et al., 2011; Krishna et al., 2016) or with anti-mitotic agents such as colchicine, trifluralin, oryzalin (Ebrahimzadeh et al., 2018), amiprofos-methyl (Kurtar et al., 2021), and carbamate (Zhou et al., 2017). Since there are no anti-mitotic agents in this experiment, TC IV could be one of the reasons for detecting polyploid, which could potentially alter the formation of chromosomal constitution and genetic materials (Debnath & Ghosh, 2022; Košmrlj et al., 2013).

A phragmoplast composed of microfilaments and microtubules forms between the daughter nuclei at the late anaphase stage, guiding the alignment and fusion of vesicles to a cell plate (Nishihama et al., 2002). Mistakes here may cause a disorganised cell plate, failure of cytokinesis and subsequently polyploidy or mixoploidy (Caetano-Pereira et al., 1998). Overexpression of specific effectors, such as the kinase-defective NPK1 and downstream effectors, disrupts phragmoplast expansion and leads to multinucleate and mixoploid cells (Eng et al., 2021; Nishihama et al., 2001). Endoreduplication, in which chromosomal DNA replication occurs without mitosis, can also alter the DNA synthesis, leading to increased ploidy levels by preceding the mitotic phase (De Almeida Engler & Gheysen, 2013; Sugimoto-Shirasu & Roberts, 2003). Besides, meiotic complications like nondisjunction can result in aneuploidy (Muenchrath et al., 2016) and, therefore, it changes the gene dosages within affected chromosomes (Potapova & Gorbsky, 2017). Normally, flow cytometry is incapable of detecting aneuploidy; thus, this condition requires both flow cytometry and karyotyping for a precise readout (Eng et al., 2021). Polyploidy or mixoploidy can also be obtained from cell fusion (syncytia) or nucleus migration (cytomixis), which generate multiple nuclei and mixed-ploidy cells (Caetano-Pereira et al., 1998). While mixoploids are generally genetically unstable, new methodologies permit ploidy sorting in chimaeras, opening the door for further research in polyploidisation (Regalado et al., 2015; Roy et al., 2001).

Ploidy can also be altered during initiation by tissue culture cellular competition. Diploid cells originating from wounded anther tissues may proliferate earlier and have advantages over haploid cells, leading to a reduction in haploid callus production. Asadi et al. (2018) recommend anther-side up position on the medium and gently scratching the sacs to promote microspore expulsion and to inhibit the diploid calli.

CONCLUSION

The present study has developed an efficient protocol for callus induction and embryo formation in *C. melo* cv. Glamour through anther culture. Of the parameters tested, 3-day cold pretreatment, 20 min surface sterilisation with 5.25% NaOCl + Tween-20, and the hormone combination of 5.0 mg/L 2, 4-D 0.5 mg/L BAP provided the highest callus, proliferation, and regeneration rates. The formation of embryogenic callus was most efficiently triggered under 4.0 mg/L BAP without the addition of auxins, and it is indicated that a high cytokinin-to-auxin ratio is important to cause the development of embryo-like structures in this species. Flow cytometric analysis demonstrated that the majority of anther-derived calli showed mixoploidy or polyploidy and that no clear evidence was found for the regeneration of haploids under the conditions tested. This aberrant ploidy variation is probably derived from TCIV and mitotic anomalies, such as abnormal cytokinesis, endoreduplication, and chromosome doubling, rather than from anti-mitotic agents. The presence of these higher ploidy levels demonstrates chromosome instability within in vitro systems and the necessity of regular cytogenetic check-ups in haploidisation programs. In general, this protocol significantly improves callus and embryo induction in *C. melo*. Although the current study has some limitations, one of them is that it stops at the embryogenic callus and embryo-like structure (ELS) stage without converting them into whole, acclimatised plantlets, which temporarily limits its practical utility in breeding programs. The frequent occurrence of mixoploidy and polyploidy during the culture cycle also challenges routine haploid or doubled haploid production. These results are useful for genetic improvement and suggest potential for the development of *C. melo* breeding programs using androgenesis-based methods.

RECOMMENDATIONS

Future research in melon breeding should follow the following directions based on the findings and limitations of this study:

Complete Plantlet Regeneration: Germination and maturation media should be optimised to facilitate the successful conversion of embryo-like structures (ELS) into fully functional shoots and roots, followed by greenhouse acclimatisation.

Refinement of Microspore Reprogramming: Investigations are needed to fine-tune the durations and intensities of physical pretreatments (such as precise cold or heat shocks) in order to increase the initial efficiency of haploid microspore embryogenesis.

Mitigation of Ploidy Instability: In order to minimise tissue culture-induced variations and somaclonal mixoploidy, research should explore the use of anti-mitotic agents at critical stages or alter culture conditions (such as altering subculture intervals and reducing 2, 4-D exposure).

Advanced Cytogenetic Screening: Future protocols should incorporate routine karyotyping alongside flow cytometry to detect aneuploidy and chromosomal rearrangements, thus verifying the genetic integrity of regenerated lines.

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REFERENCES

- Ahmadpoor, F., Zare, N., Asghari, R., & Sheikhzadeh, P. (2022). Sterilisation protocols and the effect of plant growth regulators on callus induction and secondary metabolites production in *in vitro* cultures *Melia azedarach* L. *AMB Express*, 12(1), Article 3. <https://doi.org/10.1186/s13568-022-01343-8>
- Ahmed, M. I., Fatimah, B. K., Dwi, S., Mohammed, A., & Pedram, K. (2015). Callus induction from ovules of kenaf (*Hibiscus cannabinus* L.). *Biotechnology*, 14(2), 72-78. <https://doi.org/10.3923/biotech.2015.72.78>
- Ahmed, M. I., Fatimah, B. K., Dwi, S., Pedram, K., & Mohammed, A. (2016). Haploid induction in spring onion (*Allium fistulosum* L.) via gynogenesis. *Biotechnology*, 15(1), 10-16. <https://doi.org/10.3923/biotech.2016.10.16>
- Asadi, A., Zebajadi, A., Abdollahi, M. R., & Seguí-Simarro, J. M. (2018). Assessment of different anther culture approaches to produce doubled haploids in cucumber (*Cucumis sativus* L.). *Euphytica*, 214(11), Article 214. <https://doi.org/10.1007/s10681-018-2297-x>
- Bairu, M. W., Aremu, A. O., & van Staden, J. (2011). Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regulation*, 63(2), 147-173. <https://doi.org/10.1007/s10725-010-9554-x>
- Brauen, S., Erpf, P., & Wasem, M. (2021). On absolute and relative change. *SSRN Electronic Journal*. Advance online publication, 1-10. <https://doi.org/10.2139/ssrn.3739890>
- Bridgen, M. P., Van Houtven, W., & Eeckhaut, T. (2018). Plant tissue culture techniques for breeding. In J. Van Huylenbroeck (Ed.), *Ornamental crops* (pp. 127-144). Springer. https://doi.org/10.1007/978-3-319-90698-0_6
- Caetano-Pereira, C. M., Taschetto, O. M., Pagliarini, M. S., & Brasil, E. M. (1998). Spontaneous mixoploidy in maize anthers. *Cytologia*, 63(3), 305-309. <https://doi.org/10.1508/cytologia.63.305>
- Dangi, B., Khurana-Kaul, V., & Kothari, S. L. (2014). Micropropagation of *Terminalia bellerica* from nodal explants of mature tree and assessment of genetic fidelity using ISSR and RAPD markers. *Physiology and Molecular Biology of Plants*, 20, 509-516. <https://doi.org/10.1007/s12298-014-0247-1>

- De Almeida Engler, J., & Gheysen, G. (2013). Nematode-induced endoreduplication in plant host cells: Why and how? *Molecular Plant-Microbe Interactions*, 26(1), 17-24. <https://doi.org/10.1094/MPMI-05-12-0128-CR>
- Debnath, S. C., & Ghosh, A. (2022). Phenotypic variation and epigenetic insight into tissue culture berry crops. *Frontiers in Plant Science*, 13, Article 1042726. <https://doi.org/10.3389/fpls.2022.1042726>
- Demirkaya, B., & Çömlekçioğlu, N. (2021). Effects of biotin and ascorbic acid applications on haploid embryo induction in semisolid and double layer nutrient media in pepper (*Capsicum annuum* L.) anther culture. *International Journal of Agriculture, Environment and Food Sciences*, 5(2), 191-196. <https://doi.org/10.31015/jaefs.2021.2.8>
- Deng, Y., Tang, B., Zhou, X., Fu, W., Tao, L., Zhang, L., & Chen, J. (2020). Direct regeneration of haploid or doubled haploid plantlets in cucumber (*Cucumis sativus* L.) through ovary culture. *Plant Cell, Tissue and Organ Culture*, 142(2), 253-268. <https://doi.org/10.1007/s11240-020-01839-w>
- Doležel, J., & Bartoš, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany*, 95(1), 99-110. <https://doi.org/10.1093/aob/mci005>
- Doležel, J., Greilhuber, J., & Suda, J. (2007). Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols*, 2(9), 2233-2244. <https://doi.org/10.1038/nprot.2007.310>
- Ebrahimzadeh, H., Soltanloo, H., Shariatpanahi, M. E., Eskandari, A., & Ramezanpour, S. S. (2018). Improved chromosome doubling of parthenogenetic haploid plants of cucumber (*Cucumis sativus* L.) using colchicine, trifluralin, and oryzalin. *Plant Cell, Tissue and Organ Culture*, 135(3), 407-417. <https://doi.org/10.1007/s11240-018-1473-y>
- Eng, W. H., Ho, W. S., & Ling, K. H. (2021). *In vitro* induction and identification of polyploid *Neolamarckia cadamba* plants by colchicine treatment. *PeerJ*, 9, Article e12399. <https://doi.org/10.7717/peerj.12399>
- Fitri, M., Nazar, K., Meidyawati, R., & Azmi, R. (2020). Antibacterial effect of xanthorrhizol (*Curcuma xanthorrhiza* Roxb.) against the biofilm of *Fusobacterium nucleatum*. *International Journal of Applied Pharmaceutics*, 12(2), 57-61. <https://doi.org/10.22159/ijap.2020.v12s2.PP-06>
- Golabadi, M., Ghanbari, Y., Keighobadi, K., & Ercisli, S. (2017). Embryo and callus induction by different factors in ovary culture of cucumber. *Journal of Applied Botany and Food Quality*, 90, 68-75.
- Habiba, R. (2016). Effect of genotypes, sucrose concentrations and their interaction on anther culture response on summer squash. *Journal of Agricultural Chemistry and Biotechnology*, 7(4), 113-120. <https://doi.org/10.21608/jacb.2016.40787>
- Haridhi, I. K. (2023). The effect of cold pre-treatment period on callus formation of pepper (*Capsicum annuum* L.) anther culture of local Aceh perintis genotype. *IOP Conference Series: Earth and Environmental Science*, 1183(1), Article 012040. <https://doi.org/10.1088/1755-1315/1183/1/012040>
- Hatfield, J. L., & Prueger, J. H. (2015). Temperature extremes: Effect on plant growth and development. *Weather and Climate Extremes*, 10(Part A), 4-10. <https://doi.org/10.1016/j.wace.2015.08.001>
- Ibrahim, A. M., Abbas, R. A., Aldabbagh, J. E., & Mohammed, A. (2023a). Factors affecting callus induction from anther and ovary of okra (*Abelmoschus esculentus* L.). *Indian Journal of Agricultural Research*, 57(5), 658-664. <https://doi.org/10.37077/25200860.2023.36.2.21>

- Ibrahim, A. M., Hameed, M. K., & Mohammed, A. (2023b). *In vitro* propagation of date palm (*Phoenix dactylifera* L.) cultivar Jawzi using shoot tip. *Basrah Journal of Agricultural Sciences*, 36(2), 267-284. <https://doi.org/10.37077/25200860.2023.36.2.21>
- Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant callus: Mechanisms of induction and repression. *The Plant Cell*, 25(9), 3159-3173. <https://doi.org/10.1105/tpc.113.116053>
- Košmrlj, K., Murovec, J., & Bohanec, B. (2013). Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray-irradiated pollen. *Journal of the American Society for Horticultural Science*, 138(4), 310-316. <https://doi.org/10.21273/JASHS.138.4.310>
- Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., & Sadh, R. K. (2016). Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech*, 6, Article 54. <https://doi.org/10.1007/s13205-016-0389-7>
- Kurtar, E. S., Balkaya, A., & Kandemir, D. (2016). Evaluation of haploidisation efficiency in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther culture. *Plant Cell, Tissue and Organ Culture*, 127(2), 497-511. <https://doi.org/10.1007/s11240-016-1074-6>
- Kurtar, E. S., Seymen, M., Çetin, A. N., & Türkmen, Ö. (2021). Dihaploidisation in promising summer squash genotypes (*Cucurbita pepo* L.) via irradiated pollen technique. *Yuzuncu Yil University Journal of Agricultural Sciences*, 31(1), 42-51. <https://doi.org/10.29133/yyutbd.800475>
- Mahood, H. E., Abbas, M. K., & Zahid, N. A. (2022). Micropropagation of feverfew (*Tanacetum parthenium*) and quantification of parthenolide content in its micropropagated and conventionally grown plants. *Horticulturae*, 8(1), Article 50. <https://doi.org/10.3390/horticulturae8010050>
- Mavani, H. A. K., Tew, I. M., Wong, L., Yew, H. Z., Mahyuddin, A., Ghazali, R. A., & Pow, E. H. N. (2020). Antimicrobial efficacy of fruit peels eco-enzyme against *Enterococcus faecalis*: An *in vitro* study. *International Journal of Environmental Research and Public Health*, 17(14), Article 5107. <https://doi.org/10.3390/ijerph17145107>
- Metwally, E. I., Moustafa, S. A., El-Sawy, B. I., Haroun, S. A., & Shalaby, T. A. (1998). Production of haploid plants from *in vitro* culture of unpollinated ovules of *Cucurbita pepo*. *Plant Cell, Tissue and Organ Culture*, 52(3), 117-121. <https://doi.org/10.1023/A:1005948809825>
- Midin, M. R., Nordin, M. S., Madon, M., Saleh, M. N., Goh, H., & Noor, N. M. (2017). Determination of the chromosome number and genome size of *Garcinia mangostana* L. via cytogenetics, flow cytometry and k-mer analyses. *Caryologia*, 71(1), 35-44. <https://doi.org/10.1080/00087114.2017.1403762>
- Mishra, R., & Rao, G. J. N. (2013). Effect of cold pretreatment and phytohormones on anther culture efficiency of two *indica* rice (*Oryza sativa* L.) hybrids—Ajay and Rajalaxmi. *Journal of Experimental Biology and Agricultural Sciences*, 1(2), 69-76.
- Muenchrath, D., Campbell, A., Merrick, L., & Lübberstedt, T. (2016). Ploidy—Polyploidy, aneuploidy, haploidy. In *Crop Genetics*, interactive e-learning courseware. Plant Breeding E-Learning in Africa. Retrieved from <https://pbea.agron.iastate.edu>
- Naïtchédé, L. H., Nyende, A. B., Runo, S., & Borlay, A. J. (2023). Plant regeneration from embryogenic callus-derived from immature leaves of *Momordica charantia* L. *Heliyon*, 9(11), Article e22122. <https://doi.org/10.1016/j.heliyon.2023.e22122>

- Nguyen, M. L., Ta, T. H. T., Huyen, T. N. B. T., & Voronina, A. V. (2019). Anther-derived callus formation in bitter melon (*Momordica charantia* L.) as influenced by microspore development stage and medium composition. *Sel'skokhozyaistvennaya Biologiya [Agricultural Biology]*, 54(1), 140-148. <https://doi.org/10.15389/agrobiology.2019.1.140eng>
- Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T., & Machida, Y. (2001). The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. *Genes & Development*, 15(3), 352-363. <https://doi.org/10.1101/gad.863701>
- Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., Yamazaki, Y., & Machida, Y. (2002). Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. *Cell*, 109(1), 87-99. [https://doi.org/10.1016/S0092-8674\(02\)00691-8](https://doi.org/10.1016/S0092-8674(02)00691-8)
- Ozsan, T., Gozen, V., & Onus, A. N. (2017). Cucumber gynogenesis: Effects of 8 different media on embryo and plant formation. *International Journal of Agriculture Innovations and Research*, 6(2), 419-422.
- Phillips, G. C., & Garda, M. (2019). Plant tissue culture media and practices: An overview. *In Vitro Cellular & Developmental Biology - Plant*, 55(3), 242-257. <https://doi.org/10.1007/s11627-019-09983-5>
- Potapova, T., & Gorbysky, G. J. (2017). The consequences of chromosome segregation errors in mitosis and meiosis. *Biology*, 6(1), Article 12. <https://doi.org/10.3390/biology6010012>
- Rakha, M. T., Metwally, E. I., Moustafa, S. A., Etman, A. A., & Dewir, Y. H. (2012). Evaluation of regenerated strains from six *Cucurbita* interspecific hybrids obtained through anther and ovule *in vitro* cultures. *Australian Journal of Crop Science*, 6(1), 23-30.
- Regalado, J. J., Carmona Martín, E., Castro, P., Moreno, R., Gil, J., & Encina, C. L. (2015). Study of the somaclonal variation produced by different methods of polyploidization in *Asparagus officinalis* L. *Plant Cell, Tissue and Organ Culture*, 122(1), 31-44. <https://doi.org/10.1007/s11240-015-0747-x>
- Roy, A. T., Leggett, G., & Koutoulis, A. (2001). *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). *Plant Cell Reports*, 20(6), 489-495. <https://doi.org/10.1007/s002990100364>
- Salazar-Mercado, S. A., Torres-León, C. A., & Rojas-Suárez, J. P. (2019). Cytotoxic evaluation of sodium hypochlorite, using *Pisum sativum* L. as effective bioindicator. *Ecotoxicology and Environmental Safety*, 173, 71-76. <https://doi.org/10.1016/j.ecoenv.2019.02.027>
- Simon, S., & Petrášek, J. (2011). Why plants need more than one type of auxin. *Plant Science*, 180(3), 454-460. <https://doi.org/10.1016/j.plantsci.2010.12.007>
- Skoog, F., & Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology*, 11, 118-131.
- Song, H., Lou, Q. F., Luo, X. D., Wolukau, J. N., Diao, W. P., Qian, C. T., & Chen, J. F. (2007). Regeneration of doubled haploid plants by androgenesis of cucumber (*Cucumis sativus* L.). *Plant Cell, Tissue and Organ Culture*, 90(3), 245-254. <https://doi.org/10.1007/s11240-007-9263-y>
- Song, Y. (2014). Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *Journal of Integrative Plant Biology*, 56(2), 106-113. <https://doi.org/10.1111/jipb.12131>

- Sugimoto-Shirasu, K., & Roberts, K. (2003). "Big it up": Endoreduplication and cell-size control in plants. *Current Opinion in Plant Biology*, 6(6), 544-553. <https://doi.org/10.1016/j.pbi.2003.09.009>
- Thorpe, T. A. (2007). History of plant tissue culture. *Molecular Biotechnology*, 37(2), 169-180. <https://doi.org/10.1007/s12033-007-0031-3>
- Tomaszewska, P., Pellny, T. K., Hernández, L. M., Mitchell, R. A. C., Castiblanco, V., Vega, J. J. D., Schwarzacher, T., & Heslop-Harrison, J. S. (2021). Flow cytometry-based determination of ploidy from dried leaf specimens in genomically complex collections of the tropical forage grass *Urochloa* sp. L. *Genes*, 12(7), Article 957. <https://doi.org/10.3390/genes12070957>
- Zhou, K., Fleet, P., Nevo, E., Zhang, X., & Sun, G. (2017). Transcriptome analysis reveals plant response to colchicine treatment during chromosome doubling. *Scientific Reports*, 7, Article 8503. <https://doi.org/10.1038/s41598-017-08391-2>

Review Article

Methionine, Lysine and Tryptophan Optimisation in Broiler Nutrition: Functional Roles, Supplementation Strategies and Feed Crop Biofortification

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ABSTRACT

High-quality lean tissue growth, feed efficiency, and proper immune function are essential for broiler production. Maize and soybean meal remain major ingredients in commercial broiler feeds, but their amino acid composition often does not match the dietary need for methionine (Met), lysine (Lys), and tryptophan (Trp), especially when crude protein (CP) content is lowered to improve nitrogen efficiency. In this review, the functional roles of Met, Lys, and Trp in broilers are discussed

within the context of their practical applications in feed supplementation, low-CP formulation, and long-term feed crop improvement. Met is crucial for protein synthesis, methylation, antioxidant activity, and immune function. Lys plays a critical role in muscle growth, carcass development, and shaping the ideal protein profile. Trp is involved in appetite regulation, serotonin and melatonin synthesis, stress response, and immune function. This review also addresses how the amino acid content of maize, soybean, and rice can be improved through biofortification and gene editing. It highlights that amino acid optimisation should be based on an integrated approach rather than individual

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nutrient additions. In the short term, the focus should be on balanced digestible amino acid provision and crystalline amino acid use. In the long term, crop biofortification should consider agronomy, nutritional value, economics, regulation, and consumer acceptance. Future studies should combine controlled broiler feeding trials with metabolomics, transcriptomics, microbiome, and proteomics approaches to evaluate tissue metabolism, gut health, nitrogen efficiency, and meat quality.

Keywords: Broiler nutrition, feed crop biofortification, lysine, limiting amino acids, low-crude-protein diet, methionine, proteomics, tryptophan

INTRODUCTION

Broiler production requires nutritionally balanced diets to promote rapid growth, efficient protein deposition, gut health, immune function, and high-quality meat. Contemporary broilers require significant amounts of digestible amino acids due to the rapid nature of lean tissue development during a brief production period. When amino acid provision is imbalanced, body weight gain, feed conversion ratio (FCR), breast muscle development and nitrogen utilisation may be compromised even when dietary crude protein (CP) appears adequate (Dozier et al., 2011; Maqsood et al., 2022; Maynard et al., 2022).

Plant proteins like maize and soybean meal are widely used in the preparation of commercial broiler diets. They all have their advantages and are readily available; however, the amino acids they contain are not always a perfect fit for the requirements of the broilers. Usually, methionine (Met) is the first limiting amino acid in broiler diets, especially when they are based on maize-soybean meal. Lysine (Lys) is linked to the development of lean tissue mass and has often been employed as a reference amino acid in ideal protein formulation (National Research Council, 1994; Jankowski et al., 2014; Tran et al., 2021). Tryptophan (Trp) is an essential amino acid for pigs, but with a lower requirement than Met and Lys. Nevertheless, it plays an important role in the regulation of feed intake, synthesis of serotonin, synthesis of melatonin, immune response and stress adaptation (N. Linh et al. 2021; Mund et al., 2020; Taylor-Bowden et al., 2024).

CP is an inadequate basis for formulating diets for modern broiler production since it does not indicate whether essential amino acids have a balanced and digestible level of protein molecules. Too much CP brings up feed costs, nitrogen removal and environmental effects, and the lack of any limiting amino acid will affect the ability to synthesise protein and growth. Consequently, increased emphasis should be placed on precision formulation using digestible amino acid ratios to maximise performance and minimise unnecessary nitrogen losses (Lee et al., 2020; Maynard et al., 2022; Vastolo et al., 2024).

Amino acid optimisation is mostly based on short-term use of commercially available crystalline amino acids and low-CP accurate formulations. But fluctuations in raw material costs, limited global supply and uncertainties regarding sustainability are driving the

interest in alternative long-term solutions. Maize, soybean, and rice amino acid quality can be improved intrinsically by either increasing their biosynthesis or reducing their catabolism, or altering the composition of storage proteins via feed crop biofortification, transgenic technology, or CRISPR/Cas genome editing (Devi et al., 2023; Hasan & Rima, 2021; Nagesh et al., 2024; Tien Lea et al., 2016; Tukuli, 2022). The value of these crops for broiler nutrition is not only related to their amino acid concentration but also to the amino acid digestibility, feed processing stability, agronomic performance, economic feasibility, regulatory approval, consumer acceptance, and verified broiler responses. One of the new features of this review is the comprehensive assessment of the two approaches to optimising Met, Lys and Trp in broiler nutrition, namely as short-term supplementation, and as biofortification of feed crops for inclusion in long-term supply. As opposed to many reviews, which have traditionally looked at amino acid nutrition and crop improvement separately, this review synthesises current knowledge on broiler physiological requirements, feed formulation practices, and the genetic improvement of plant-based feed ingredients. It takes a comprehensive approach which provides a framework for improving broiler performance while minimising nitrogen losses and sustainable broiler production.

REVIEW METHODOLOGY

A narrative review was undertaken based on peer-reviewed literature from Scopus, Web of Science, PubMed, ScienceDirect, Google Scholar and specific publishers' databases. The search strategy included various combinations of the following terms: broiler, methionine, Lys, trypto-phan, limiting amino acids, digestible amino acids, ideal protein, feed conversion ratio, low crude protein diet, amino acid supplementation, broiler immunity, antioxidant defense, mTOR, AMPK, feed crop biofortification, maize, soybean, rice, CRISPR, genetic engineering, transgenic crops, proteomics, metabolomics, and gut microbiome. Peer-reviewed studies that were published from 2014 to 2025 were prioritised, and foundational references were included where applicable, such as the NRC recommendations and previous broiler amino acid studies (National Research Council, 1994; Dozier et al., 2011; Vazquez-Anon et al., 2006). Studies were included if they focused on broiler amino acid requirements, growth performance, FCR, carcass characteristics, immune response, oxidative status, metabolic signalling, feed formulation, low crude protein (low-CP) diets or crop-based amino acid enhancement. Non-broiler species studies were not considered as primary nutritional evidence and were included only when they provided direct mechanistic support to the amino acid metabolism, crop biofortification or omics interpretation.

The literature search and synthesis process is summarised in Figure 1. The evidence was sorted into the following categories: amino acid requirements of broilers, function of Met, Lys, and Trp in broilers, integrated metabolic function, short-term supplementation strategies, biofortification of feed ingredients, sustainability considerations, and future

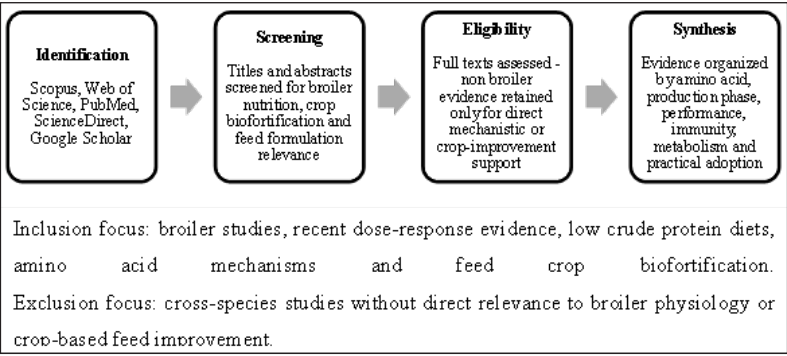


Figure 1. The literature search and synthesis workflow employed in this review prioritises broiler-specific evidence and includes crop biofortification or mechanistic studies only when they are directly relevant to broiler feed formulation

proteomics-based validation. Such an organisation helps to keep broiler nutrition a matter of prime interest and to consider the long-term importance of feed crop improvement.

THE IMPORTANCE OF ADDRESSING LIMITING AMINO ACIDS IN BROILER FEED FORMULATION

Limiting amino acids are those in the lowest amount in comparison with the nutritional requirements of the bird. An inadequate proportion of any essential amino acid will limit protein synthesis, even if the amount of crude protein and the other amino acids are adequate. Met, Lys and Trp control complementary biological processes in broiler diets. Met is involved in the metabolism of sulphur amino acids, methylation, antioxidant defence and immune function, whilst Lys is required for lean tissue deposition and muscle protein accretion. Conversely, Trp is involved in appetite regulation, synthesis of serotonin and melatonin, stress response and modulation of immune function (Jankowski et al., 2014; Khwatenge et al., 2020; N. T. Linh et al., 2021; Song et al., 2021).

One of the important consequences of this for broiler diet formulation is that amino acid needs should be evaluated as a part of a balanced digestible amino acid profile, not as a single nutrient. If only one of the limiting amino acids is supplemented without the other limiting amino acids being altered, the growth response will be variable because growth will be limited by the next limiting nutrient. This is especially critical in low-CP diets, where the amount of intact protein is decreased, and the requirement for supplemental crystalline amino acids becomes more critical, and the supply of non-essential amino acids must be carefully considered (Lee et al., 2020; Maynard et al., 2022; Maqsood et al., 2022).

Amino acid optimisation is also very practical and environmentally friendly. Properly balanced diets can lead to better FCR, maintain the carcass yield, decrease overfeeding of N and decrease N excretion. Optimum levels, however, depend on the strain, sex, age, health status, environmental stress, ingredient composition and the criterion of response,

such as body weight gain, FCR, breast meat yield, immune response and/or oxidative status (Chang et al., 2024; Macelline et al., 2021; Mund et al., 2020; Tran et al., 2021). The requirement values and biological basis for variation among studies are discussed in the following section.

METHIONINE IN BROILER NUTRITION: PROTEIN SYNTHESIS, ANTIOXIDANT DEFENSE AND IMMUNITY

Methionine is generally considered the first limiting amino acid in the maize-soybean meal broiler ration. Based on NRC recommendations, starter diets should have about 0.50% Met and grower diets about 0.38% Met, but practical requirements will depend on the strain, age, dietary CP, total SAA intake and response endpoint (National Research Council, 1994; Jankowski et al., 2014). Starter diets need to promote muscle growth, immune competence, intestinal maturation and organ development, and adequate Met supply is essential during early growth.

Met is a precursor of homocysteine, creatine, carnitine, growth and cell metabolism metabolites, and is a contributor to protein synthesis (Ramadan et al., 2021). This is because its functions are not limited to growth; sulfur amino acid metabolism plays a key role in glutathione production and antioxidant defence. Additionally, Met deficiency not only causes damage to the liver and kidneys but also increases the oxidative stress in the blood and weakens the immune function of the ileal mucosa in broilers (Song et al. 2021). These effects highlight the need to assess Met response, along with growth performance and antioxidant, immune and intestinal parameters.

DL-methionine, L-methionine or 2-hydroxy-4-(methylthio)butanoic acid (HMTBa) is the typical source of supplemental Met. Amino acid digestibility at the ileum, broiler growth performance, and body weight gain may be affected by graded inclusion of Met, and granulated L-methionine can be used to support broiler growth performance equivalent to DL-methionine (Macelline et al., 2021; Yoon et al., 2024). While growth promotion is a potential benefit of HMTBa, its relative effectiveness can depend on the dose used, basal diet composition and evaluation criteria (Calderon-Ardila et al., 2024; Vazquez-Anon et al., 2006). Thus, source selection must take into account the bioefficacy, feed manufacturing conditions, cost and specific feed production goals.

Reported Met responses are not necessarily uniform. Benefits from improvement in growth, intestinal function and antioxidant defence are most pronounced when the basal diet is sulfur amino acid deficient or marginal, but the addition of Met in excess of requirement may fail to further improve body weight and/or feed conversion ratio. This variation might be due to differences in breed/strain, phase of production, basal SAA content, environmental stress, and whether or not the study included immune and oxidative stress responses. When formulating broilers, it is better to aim for a sufficient digestible sulfur amino acid balance instead of maximum Met.

LYSINE IN BROILER NUTRITION: MUSCLE DEVELOPMENT, FEED EFFICIENCY AND MEAT QUALITY

Lysine is closely related to protein deposition and lean tissue growth and is important in broiler muscle accretion, breast meat yield, carcass development and feed efficiency. The National Research Council (1994) suggest 1.10% Lys in starter diets, 1.00% Lys in grower diets and 0.85% Lys in finisher diets as recommended by the NRC. The digestible Lys is often used as the reference amino acid for ideal amino acid ratios when formulating diets for today's broilers.

Lys can help promote muscle growth by aiding in protein synthesis, carnitine production, nutrient transport and appetite regulation. Lys-mediated neuropeptide responses have been linked to satiety and feed intake regulation in broilers, indicating that Lys can impact nutrient partitioning and feeding behaviour (Khwatenge et al., 2020; Wang & Bin, 2024). An adequate supply of Lys also contributes to myofiber development and improves carcass traits, important commercial attributes in broiler production (Chang et al., 2024; Zhang et al., 2024).

Existing evidence shows that genotype and the production phase have a strong impact on Lys response. Generally, fast-growing broilers are more sensitive to dietary Lys levels than slow-growing broilers, because they deposit more lean tissue than slow growers (Tran et al., 2021). Lys deficiency or excess affects growth and also the expression of genes related to lipid metabolism, thus showing that Lys balance affects protein accretion and nutrient partitioning (Tian et al., 2019). As a consequence, the Lys recommendations should depend on age, genotype, dietary energy level, digestible amino acid balance, as well as production goals like growth rate, FCR, breast yield or meat quality.

One important practical aspect is that FCR may be optimised at a different level of Lys than breast meat yield and development of the myofibers. Thus, total Lys concentration should not be the only criterion for diet formulation. To ensure that digestible Lys matches the production stage, it should instead serve as the reference amino acid for balancing Met, Trp, threonine, valine, and other amino acids in low-CP diets (Lee et al., 2020; Maynard et al., 2022; Maqsood et al., 2022).

TRYPTOPHAN IN BROILER NUTRITION: APPETITE REGULATION, STRESS RESPONSE AND IMMUNITY

Tryptophan is an indispensable amino acid that is essential for broiler growth, appetite control, the immune system and adaptation to stress. In poultry, Trp is a precursor for the synthesis of serotonin, melatonin, and niacin, associating amino acid provision with neuroendocrine function, behaviour, antioxidant defence, and immune regulation (N. T. Linh et al., 2021; Mund et al., 2020; Taylor-Bowden et al., 2024). Trp is generally an amino acid that is less required than Met and Lys, but is more important during stress or immune challenge, heat stress or low CP diets.

Findings in broilers suggest that supplementing Trp, especially under physiological stress or when the basal diet is low in Trp, may improve growth performance, immune status and antioxidant status (N. T. Linh et al., 2021). Thus, Trp must not only be seen as a growth-promoting nutrient, but as a functional amino acid that helps to stabilise behaviour, adapt to stress and to enhance immune competence.

The reported Trp requirements vary, depending on the measured response, diet composition, genetic strain and age. Based on the recommendation of the National Research Council (NRC), the level of Trp in starter and grower diets should be 0.20% and 0.17%, respectively. But, some poultry studies have recorded results at the same or slightly higher concentrations, depending on the dietary and production conditions (National Research Council, 1994; Lisnahan & Nahak, 2020; Mund et al., 2020). This variation reinforces the necessity of understanding the Trp requirements with a digestible amino acid balance, crude protein level and environmental stress, as well as the desired response.

Mechanistic and functional studies in pigs, ruminants, or fish might give some clues towards the mechanism of metabolism of Trp or serotonin signalling, but could not replace direct evidence from broiler feeding trials. Thus, the inclusion of Trp optimisation in optimal protein formulation strategies should be considered, especially when decreasing CP or during stress-induced reduction in feed intake and immune stability.

INTEGRATED METABOLIC ROLES OF METHIONINE, LYSINE AND TRYPTOPHAN

Met, Lys and Trp operate in different, but complementary ways. Met and Lys are closely linked to protein synthesis and anabolic signalling of mTOR, and Trp is associated with the production of serotonin and melatonin, regulation of appetite and AMPK-mediated energy sensing (Hu et al., 2021; Klann et al., 2020; Sharma et al., 2023). The physiological responses to these amino acids should not be considered as isolated feed components, but should depend on the level of the other amino acids in the feed.

While protein deposition and antioxidant protection are promoted by Met, its use is limited if protein growth is limited due to a shortage of Lys. Although Lys is important for muscle accretion, responses to Lys supplementation are only minimal if the supply of SAA is inadequate. The effects of Met and Lys on performance may be influenced by Trp-mediated effects on feed intake, stress adaptability and immune competence. This integrated perspective is particularly true in low-CP foods where amino acid balance becomes much more important and where excess CP will not correct for an amino acid deficiency (Lee et al., 2020; Maynard et al., 2022).

In this integrated framework, the functional roles of Met, Lys and Trp are linked to practical outcomes in broiler's diets (Figure 2). The figure demonstrates that amino acid optimisation extends beyond supplementation, encompassing formulation and sustainability considerations such as digestible amino acid balance, low-CP diets, crop improvement, and validation through animal performance responses.

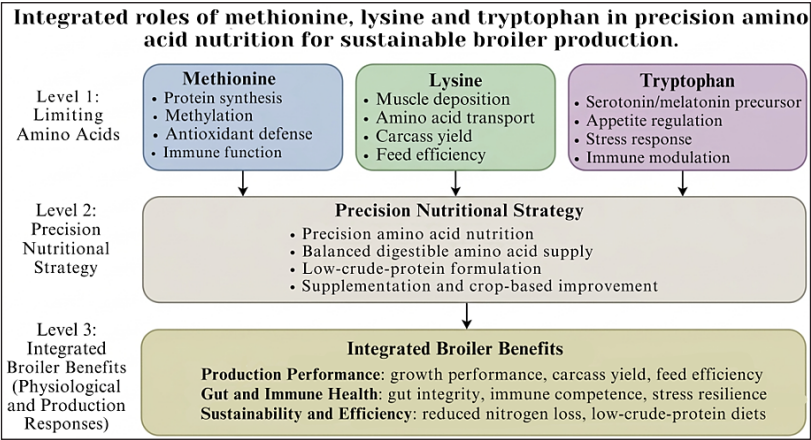


Figure 2. Integrated approach to optimise Met, Lys and Trp in broiler rations. This figure illustrates that practical formulation should be directed towards providing a balance of digestible amino acids rather than raising individual amino acids in isolation

Met, Lys and Trp requirements vary with broiler age, broiler genotype, growth rate, basal diet composition, CP content, environment and the criterion for adequacy. Although NRC values are good benchmarks, the modern broiler formulation needs to be flexible as requirement estimates depend on the specific production trait being considered, such as body weight gain, FCR, immune response, antioxidant status, muscle development, and carcass traits (National Research Council, 1994; Jankowski et al., 2014; Macelline et al., 2021; Mund et al., 2020; Tran et al., 2021). The levels of requirements and some of the results reported are summarised in Table 1 to indicate the necessity of formulating amino acids on a phase basis and not as a fixed percentage of the diet.

Direct comparison of biological function, mechanism of action, deficiency effects, and dietary formulation implications of Met, Lys, and Trp can help elucidate the importance of these amino acids. All three are essential amino acids, but they affect broiler performance by different biological mechanisms. Table 2 summarises these differences and links them to practical broiler feed formulation.

As shown in Table 2, the growth of broiler chickens is impaired by Met deficiency, and their antioxidant and immune protection is reduced. Thus, Met is considered a major limiting amino acid in maize-soybean meal-based diets (Song et al., 2021; Taylor-Bowden et al., 2024). The major effects of Lys imbalance on livestock production are on lean tissue deposition, feed efficiency, breast muscle development and carcass quality, and digestible Lys is one of the key protein amino acids used in amino acid ratio formulation (Khwatenge et al., 2020; Tian et al., 2019; Tran et al., 2021). Due to its importance as a precursor for serotonin and melatonin, deficiency in Trp may lead to decreased intake and reduced resistance to stress, inferior behaviour, and an impact on the immune system (N. T. Linh et al., 2021; Mund et al., 2020). Thus, the data in Table 2 justify the contention that digestible

Table 1
Reported Met, Lys, and Trp requirement levels in broiler diets according to NRC recommendations and selected broiler studies

Amino Acid	NRC Recommendation (% of Diet)	Selected Reported Findings	Interpretation for Broiler Formulation	Key References
Met	Starter: 0.50; grower: 0.38	Graded Met inclusion affects growth performance and ileal amino acid digestibility; L-Met and DL-Met can support comparable broiler growth under appropriate formulation.	Prioritise adequate digestible sulfur amino acid balance during early growth, antioxidant defence, and immune support; avoid unnecessary excess beyond requirement.	National Research Council, 1994; Jankowski et al., 2014; Macelline et al., 2021; Song et al., 2021; Yoon et al., 2024
Lys	Starter: 1.10; grower: 1.00; finisher: 0.85	Responses vary by genotype and phase: Lys influences muscle accretion, myofiber development, carcass traits and feed efficiency.	Use digestible Lys as the reference amino acid for ideal protein formulation- align with growth rate, carcass target and diet phase.	National Research Council, 1994; Fijalovych et al., 2022; Tran et al., 2021; Chang et al., 2024
Trp	Starter: 0.20; grower: 0.17	Supplementation can affect growth performance, immune response, antioxidant status and intestinal morphology, with responses depending on diet and age.	Balance Trp carefully in low: CP diets and stress-related conditions because it affects appetite, behaviour, serotonin/melatonin pathways and immunity.	National Research Council, 1994; Mund et al., 2020; N. T. Linh et al., 2021; Lisnahan & Nahak, 2020

Note. Values should be interpreted in relation to digestible amino acid formulation, production phase, genotype, basal diet and response endpoint

amino acid balances rather than minimum digestible amino acid requirements should be the criteria used for formulating broiler diets.

SHORT-TERM AMINO ACID OPTIMISATION STRATEGIES IN BROILER DIETS

Short-term strategies are those that are applicable to immediate use by feed formulators and commercial producers. The most convenient solution is to use crystalline amino acid supplementation, because Met, Lys and Trp are commercially available and can be precisely added. This supplementation allows CP reduction while keeping a digestible amino acid balance and boosting nitrogen efficiency, leading to a lower nitrogen excretion (Lee et al., 2020; Maynard et al., 2022; Vastolo et al., 2024).

Low-CP diets are a critical part of environmental sustainability. But, when CP is reduced, while keeping the amino acid profile at the optimum level, growth, FCR and carcass composition may be affected. So, reduction of CP should go hand in hand with proper percentages of digestible Met, Lys, Trp and other limiting amino acids, digestibility

Table 2
Functional roles, mechanisms and practical implications of Met, Lys and Trp in broiler nutrition

Amino Acid	Main Functions in Broilers	Key Pathways or Mechanisms	Deficiency or Imbalance Effect	Practical Implication
Met	Protein synthesis, methylation, cysteine and glutathione formation, antioxidant defence, immune support and gut integrity.	Sulfur amino acid metabolism, methyl donor pathways, glutathione-related antioxidant defence, and mTOR-associated protein synthesis.	Reduced growth, impaired antioxidant defence, weakened mucosal immunity and poorer liver/kidney health.	Balance digestible sulfur amino acids, especially in starter and low-CP diets; select a source based on bioefficacy and cost.
Lys	Lean tissue deposition, breast muscle development, carcass traits, feed efficiency and appetite-related signalling.	Ideal protein reference amino acid, protein accretion, nutrient transport, and neuropeptide-associated feed intake regulation.	Reduced protein accretion, altered feed intake, impaired carcass development and poorer meat quality response.	Use digestible Lys as the main reference for amino acid ratios and adjust by genotype, phase and carcass objective.
Trp	Feed intake regulation, serotonin and melatonin production, stress resilience, behaviour, antioxidant status and immune modulation.	Serotonin/melatonin/niacin metabolism; AMPK-related energy sensing; neuroendocrine regulation.	Lower feed intake, poorer stress resilience, impaired immune response and inconsistent growth.	Prioritise in low-CP diets and stress-prone conditions where appetite, behaviour, and immunity influence performance.

of the ingredients, enzymes and overall health status (Maqsood et al., 2022; Maynard et al., 2022).

In practical terms, Met and Lys are the most important amino acids to consider in most maize-soybean broiler diets, directly related to protein deposition and growth performance. When diets are formulated with lower CP levels, or when birds are subjected to stressors that affect their appetite, behaviour and immune function, the importance of Trp increases. A dietary approach is suggested for each phase. Starter diets should be designed to assist in early growth and immune system development, grower diets to ensure that lean tissue accretion is efficient, and finisher diets to ensure that carcass yield and nitrogen efficiency are accomplished as low a cost possible.

FEED CROP BIOFORTIFICATION AS A LONG-TERM STRATEGY

Biofortification of feed crops is a long-term approach towards improving the inherent nutritional quality of plant-based ingredients. Amino acid levels can be enhanced in crops like maize, soybean, and rice, through the increase of biosynthetic flux, reduction of catabolism, alteration of storage protein, or the incorporation of amino acid-rich proteins (Devi et al., 2023; Hagan & Higgins, 2024; Nagesh et al., 2024; Tien Lea et al., 2016). Such methods will be especially applicable to broiler nutrition, which could help decrease

the need for an external source of synthetic amino acids and enhance the base quality of the feed ingredients.

The biological theory behind biofortification of feed crops is supported by the understanding of plant metabolic pathways involved in the amino acid biosynthesis and accumulation. Lys and Met are both associated with the pathway of amino acid biosynthesis from the aspartate family of amino acids, which also generates threonine and isoleucine (Han et al., 2021; Tien Lea et al., 2016). Increasing the concentration of one target amino acid may affect the accumulation of other amino acids because they share common precursors and regulatory enzymes, including feedback inhibition, changes in carbon and nitrogen allocation, and competition between branch pathways (Yang et al., 2020; Zafar & Jianlong, 2023). Figure 3 shows the biochemical background and the importance of taking the whole amino acid balance into account when improving crops, instead of only targeting one specific amino acid.

As illustrated in Figure 3, aspartate is a central precursor for amino acids, which are essential for feeding improvement of feed crops such as Lys, Met, threonine and isoleucine. These are enzymes that could be targeted for genetic engineering, marker-assisted selection or CRISPR/Cas mediated editing, such as aspartate kinase (Devi et al., 2023), dihydrodipicolinate synthase (Nagesh et al., 2024), threonine synthase, cystathionine gamma-synthase, Met synthase and Met gamma-lyase (Duo et al., 2024; Hasan, 2024; Tukuli, 2022). But when this pathway is modified, it may lead to some compromises like changes in the seed composition, loss in germination, lower yield or imbalance in the related amino acids (Bhargava et al., 2024; Yang et al., 2020; Zafar & Jianlong, 2023). Therefore, it is important to assess biofortified crops for digestible amino acid availability, ingredient quality, anti-nutritional effects, feed processing stability, and actual growth performance when considering them for broiler nutrition.

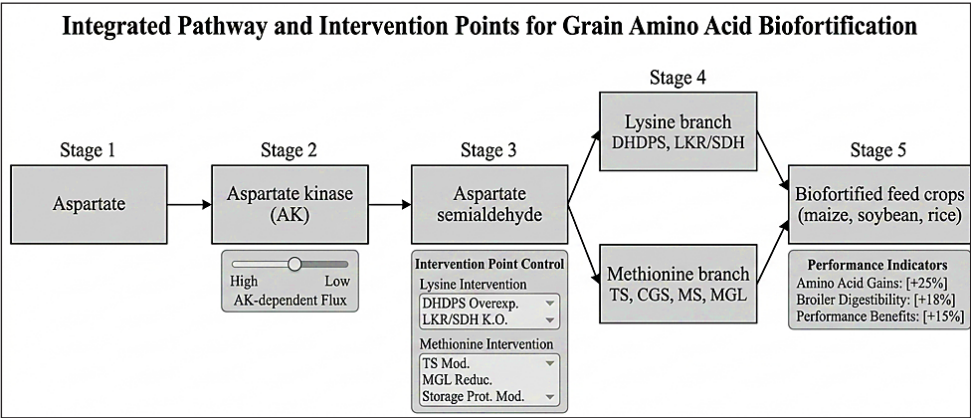


Figure 3. The simplified aspartate-derived amino acid pathway is relevant to feed crop biofortification

Several strategies for crop biofortification have been suggested to enhance the amino acid composition of plant feed for broilers. These approaches are especially relevant as maize and soybean meal are commonly used ingredients in commercial broiler diets and can be low in Met and Lys when feeding for high nitrogen efficiency and rapid growth (Tien Lea et al., 2016; Te Pas et al., 2021; Pope et al., 2024). Lys enhancement has been done in the following ways, as summarised in Table 3: 1) via quality protein maize; 2) via opaque2 and opaque16; 3) via manipulation of dihydrodipicolinate synthase; 4) via alpha-zein suppression; and 5) via reduced Lys catabolism (Chhabra et al. 2024; Dixit et al. 2021; Wu et al., 2024).

Table 3

Feed crop biofortification strategies for improving amino acid profiles and their relevance to broiler feed formulation

Crop or Strategy	Target Amino Acid	Genetic or Metabolic Approach	Reported Outcome or Rationale	Relevance to Broiler Feed Formulation
Quality protein maize and opaque2/opaque16 strategies	Lys and Trp	Modification of zein protein profile through opaque2-based quality protein maize and related modifier loci.	Improves protein quality and may increase Lys and Trp concentration in maize grain.	Directly relevant because maize is a major broiler feed ingredient, and Lys supports muscle accretion.
DHDPS manipulation, CordapA expression and reduced Lys catabolism	Lys	Feedback-insensitive dihydrodipicolinate synthase expression and/or suppression of Lys catabolism.	Can increase seed Lys accumulation but may require careful control to avoid agronomic penalties.	Potential long-term strategy to reduce reliance on supplemental Lys if digestibility and crop performance are validated.
Alpha-zein suppression or storage protein rebalancing in maize	Lys	Downregulation or alteration of Lys-poor zein fractions to improve relative Lys content.	Can improve the amino acid profile, although seed texture and yield must be monitored.	May improve maize protein quality, but feeding value must be tested in broiler diets.
Met-rich maize improvement	Met	Manipulation of sulfur assimilation, Met biosynthesis, Met-rich zeins and related QTL.	May increase sulfur-containing amino acids and improve intrinsic Met supply.	Relevant because Met is often first limiting in maize-soybean meal broiler diets.
Met-enhanced soybean	Met	Expression of Met-rich proteins, modification of storage proteins, and CRISPR/Cas targeting of Met catabolism, such as MGL.	May increase seed Met concentration, although protein balance and agronomic stability must be confirmed.	Could improve sulfur amino acid supply from soybean meal if digestibility, processing stability and safety are validated.
Rice and other model or staple crops	Lys and Met	Lys-rich protein expression, altered Lys catabolism, sulfur pathway manipulation and genome-wide trait identification.	Useful for understanding pathway regulation and possible biofortification tools.	Indirect relevance to broiler feed unless used as an ingredient or as a model for maize/soybean improvement.

The use of Met-rich protein expression, changing sulfur assimilation process, altering Met biosynthesis process, and CRISPR/Cas mediated downregulation of Met synthesis or rebalancing of storage proteins are all approaches that have been taken to enhance met (Devi et al., 2023; Duo et al., 2024; Maqbool et al., 2021; Nagesh et al., 2024; Tukuli, 2022).

When evaluating feed crop biofortification, Table 3 summarises that this should not be considered as a direct replacement for the supplementation of crystalline amino acids but rather as a complementary approach, which is more aptly achieved as a long-term strategy. Some of the most directly relevant approaches include high-Lys maize and quality protein maize, given the widespread use of maize in broiler diets, and the close relationship of Lys with muscle accretion, carcass development and feed efficiency (Chang et al., 2024; Maqbool et al., 2021; Tran et al., 2021). Met-enriched soybean and maize are also highly recommended since Met is frequently the first limiting amino acid in maize-soybean meal-based broiler diets, and it is strongly associated with protein synthesis, antioxidant defence and immune function (Devi et al., 2023; Jankowski et al., 2014; Nagesh et al., 2024; Song et al., 2021). But just increasing the amount of amino acids in the seed is not enough. It should also ensure good agronomic performance, seed quality, digestibility of the nutrients, feed processing stability, and economic viability, and comply with regulatory and consumer acceptance standards (Naveen & Sontakke, 2024; Sheoran et al., 2022).

PRACTICAL, ECONOMIC, ENVIRONMENTAL AND ETHICAL CONSIDERATIONS

For commercial broiler production, the most convenient and feasible short-term means of action is not to abandon synthetic amino acids but to utilise them optimally. Crystalline amino acids allow for the formulation of low CP diets, reduce reliance on excess whole protein and can improve accuracy in meeting digestible amino acid requirements. However, there are limitations such as costs, availability, and susceptibility to global feed additive supply chain disruption, as well as high-quality soybean meal in regions where the commodity is expensive (Pope et al., 2024; Vastolo et al., 2024).

One of the main reasons for changing from a CP-based formulation to an amino acid-balanced formulation is for environmental sustainability. High feed CP levels increase nitrogen intake and excretion, leading to extra ammonia emissions and nutrient loading. Protein quality balanced for digestible Met, Lys, Trp and other limiting amino acids can support animal performance at lower CP levels, provided that the diet is correct. But, high levels of CP reduction without adequate amino acid and nitrogen balance can have adverse effects on growth, gut health or carcass quality (Lee et al., 2020; Maqsood et al., 2022; Maynard et al., 2022). Biofortified and gene-edited feed crops offer great potential in the long term, but are also subject to regulatory, ethical, and consumer acceptance issues. To achieve widespread adoption, transparent safety assessments, consistent agronomic

performance, feed industry acceptance, clear labelling policies, consumer confidence, and compelling evidence supporting the improved nutritional and ecological outcomes of these crops must all be present (Naveen & Sontakke, 2024; Sheoran et al., 2022). Ethical considerations should also take into account equitable access - expensive proprietary crops may not be a good option for small farmers or resource-poor areas.

One alternative is to use a mixed strategy, which is a pragmatic solution. Existing feed additives should be optimised in the short term to achieve precision supplementation and to provide low-CP diets. Biofortified crops should be considered as complementary foods in the medium to long-term. These strategies have been summarised according to the time frame of implementation, practicability, limitations, and suitability for broiler production in Table 4.

Table 4
Short-term and long-term amino acid optimisation strategies for sustainable broiler production

Strategy	Timeframe	Practical Value	Main Limitation	Best use in Broiler Production
Crystalline Met, Lys and Trp supplementation	Short-term	Commercially available, precise and compatible with the least-cost formulation.	Cost and supply-chain dependence require an accurate digestible amino acid matrix.	Immediate correction of limiting amino acids, especially in maize-soybean meal diets.
Low-CP precision diets	Short-term	Can reduce nitrogen intake and excretion while maintaining performance.	Risk of poor growth if the amino acid balance, non-essential nitrogen or electrolyte balance is inadequate.	An environmental strategy, when supported by a balanced digestible amino acid formulation.
Improved ingredient evaluation and enzyme support	Short- to medium-term	Improves confidence in digestible amino acid values and nutrient release.	Responses vary by ingredient quality, processing and enzyme-substrate match.	Refining formulation accuracy and reducing safety margins.
Feed crop biofortification	Long-term	Improves the intrinsic amino acid quality of plant-based ingredients.	Requires agronomic stability, digestibility confirmation, feeding trials and adoption pathways.	Complementary ingredient strategy to reduce long-term supplementation pressure.
CRISPR/ Cas and transgenic crop improvement	Long-term	Allows targeted changes in biosynthesis, catabolism or storage protein composition.	Regulatory, ethical, consumer acceptance and intellectual property challenges.	Development of amino acid-enhanced maize, soybean and other feed crops.
Proteomics and multi-omics-guided precision feeding	Research to application	Explains tissue-level responses and supports biologically grounded formulation.	Requires technical capacity, cost and integration with performance data.	Future validation of diet, ingredient and genotype-specific amino acid responses.

Table 4 highlights that only one amino acid strategy will not resolve all the problems in amino acid optimisation. At present, the use of crystalline amino acid supplementation is the most convenient and realistic short-term option as it can be very accurate, is available commercially and can be easily incorporated into low CP diets. Low-CP diets can lower N excretion, provided that Met, Lys, Trp and other potentially limiting amino acids are properly balanced (Lee et al., 2020; Maynard et al., 2022; Vastolo et al., 2024). Feed crop biofortification and CRISPR/Cas-based crop improvement can help to decrease the need for external supplementation in the future, but these methods face challenges such as agronomic, regulatory, and ethical and consumer acceptance issues (Devi et al., 2023; Naveen & Sontakke, 2024; Tukuli, 2022). Proteomics and multi-omics-guided feeding should be future research-to-application tools, since they can explain what happens on the tissue level as a result of the amino acid supply and support, including a biological basis for precision nutrition models, which are based on performance endpoints only (Klünemann et al., 2024; Wang & Bin, 2024).

FUTURE RESEARCH PRIORITIES: PROTEOMICS AND MULTI-OMICS VALIDATION

Future studies should be conducted to better understand the mechanisms through which amino acid optimum affects broiler physiology using an integrated omics approach instead of growth performance only (Rajah Kumar et al., 2025). Breast muscle, liver, intestine and immune tissues can be subjected to quantitative proteomics to detect changes in protein synthesis, amino acid transport, mitochondrial metabolism, oxidative defence, inflammatory signalling, and tissue remodelling. This is significant as two diets could produce the same weight gain in the animal, but trigger different molecular mechanisms that impact gut integrity, meat quality, or immune function (Klünemann et al., 2024; Taylor-Bowden et al., 2024).

Proteomics should be used alongside transcriptomics, metabolomics and microbiome profiling to provide a complete picture of the broiler response (Che Zahari et al., 2026). The use of transcriptomics techniques can be used to show whether gene expression has changed in response to the provision of amino acids, and the use of metabolomics techniques can quantify changes in sulfur amino acid metabolism, serotonin-melatonin pathways, nitrogen utilisation, and energy balance. Microbiome analysis can be used to determine if amino acid-balanced feeding programs or biofortified crops change the gut microbiome (Kwak et al., 2024; Taylor-Bowden et al., 2024; Wang & Bin, 2024). The combined results of these methods may help explain the different amino acid requirements for different broiler strains, stages of production, types of diet and environmental conditions. Validation of crop-feed-animal systems is a large future research gap. Seed amino acid level, amino acid digestibility, feed processing stability, influence on gut microbiota, impact on immune function, meat quality and long-term production performance are important parameters to

consider when evaluating biofortified feed crops. Comparative trials should be conducted between biofortified ingredients and conventional ingredients fortified with crystalline amino acids, to assess if and what the benefits are from crop biofortification of a nutritional, economic or environmental nature.

Further studies are also needed to determine breed and phase-specific amino acid requirements. Optimum digestible Lys, sulfur amino acids, and Trp levels could be different for fast-growing broilers as compared to slow-growing broilers. Further, the starter, grower and finisher phases should be assessed separately since early nutrient availability will influence organ development and later performance. Should include cost-benefit analysis and environmental N output assessment to ensure commercial viability.

CONCLUSION

The optimisation of Met, Lys and Trp in broiler nutrition should be considered a whole nutrition strategy, rather than a series of amino acid supplementation. Met is important for protein synthesis, methylation, antioxidant protection and immune function. Lys is needed for the growth of the carcass and muscle. Trp plays a role in the regulation of appetite, stress resistance and immune modulation. The utility of these amino acids depends upon a balance between digestible amino acids, stage of production and desired biological response.

In the short term, precision formulation using digestible amino acid ratios and supplementing with crystalline amino acids is still the best way to maximise feed efficiency and minimize nitrogen excretion. Feed crop biofortification and gene-editing technologies potentially can improve the inherent amino acid content of plant-based feed ingredients over the longer-term. Evidence of agronomic stability, proven benefits to broiler performance, feed safety, economic feasibility, regulatory approval and consumer acceptance are needed, however, for the adoption of these technologies. In future studies, amino acid strategies should be biologically sound, profitable and environmentally friendly, as well as based on the combination of controlled broiler feeding studies, proteomics, metabolomics and studies on the gut microbiome.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

CNMCZ conducted technical validation and collected data. CNMCZ & NBMA performed statistical analysis and drafted the manuscript. NMANAR supervised the project and was involved in experimental design. CNMCZ, NMS, NAB, and MAO conceptualised the study, drafting and revising the manuscript. ALTC and SRAU commented on previous versions of the manuscript. All authors read and approved the final manuscript.

REFERENCES

- Bhargava, K., Bhavana, P., Gadag, R. N., Gowtham, K., Hemalatha, N., & Kumar, G. A. (2024). Breeding strategies for quality protein maize: A review. *Emergent Life Sciences Research*, 10(1), 19-32. <https://doi.org/10.31783/elr.2024.1011932>
- Calderon-Ardila, S., Morvan, D., Péruch, O., Bellière-Baca, V., Dusselier, M., & Sels, B. F. (2024). Methionine and its hydroxy analogues: The paths toward their sustainable chemical synthesis. *Green Chemistry*, 26(8), 4242-4269. <https://doi.org/10.1039/D3GC03826B>
- Chang, C., Zhao, W., Zhang, Q., Wang, X., Zhang, J., Yan, Z., Cao, J., Liu, H., & Geng, A. (2024). Dietary crude protein and lysine levels affect meat quality and myofiber characteristic of slow-growing chicken. *Animals*, 14(14), Article 2068. <https://doi.org/10.3390/ani14142068>
- Che Zahari, C. N. M., Nik Abd Rahman, N. M. A., Mohamad Yusman, P. Q., Zaladi, N. A., Md Saad, N., Ahmad Usuldin, S. R., Mohammad, M., & Leow, A. T. C. (2026). Optimisation of protein extraction and functional proteome profiling of *Trichanthera gigantea* reveals metabolic adaptations for tropical resilience. *Journal of the Saudi Society of Agricultural Sciences*, 25(5), 332-343. <https://doi.org/10.1007/s44447-026-00168-2>
- Chhabra, G., Kaur, G., Singh, K., Kaur, J., Praba, U. P., Singh, R., Karnatam, K. S., Garg, T., Ranjan, R., & Vikal, Y. (2024). Biofortification of maize: A promising approach for better nutrition. In S. Kumar & P. S. Shukla (Eds.), *Harnessing crop biofortification for sustainable agriculture* (pp. 145-178). Springer. https://doi.org/10.1007/978-981-97-3438-2_8
- National Research Council. (1994). *Nutrient requirements of poultry* (9th rev. ed.). (pp. 1-157). The National Academies Press. <https://doi.org/10.17226/2114>
- Devi, V., Bhushan, B., Gupta, M., Sethi, M., Kaur, C., Singh, A., Singh, V., Kumar, R., Rakshit, S., & Chaudhary, D. P. (2023). Genetic and molecular understanding for the development of methionine-rich maize: A holistic approach. *Frontiers in Plant Science*, 14, Article 1249230. <https://doi.org/10.3389/fpls.2023.1249230>
- Dixit, S., Shukla, A., Singh, V., & Upadhyay, S. K. (2021). Engineering of plant metabolic pathway for nutritional improvement: Recent advances and challenges. *Genome Engineering for Crop Improvement*, 351-379
- Dozier, W. A., Corzo, A., Kidd, M. T., Tillman, P. B., & Branton, S. L. (2011). Determination of the fourth and fifth limiting amino acids in broilers fed diets containing maize, soybean meal and poultry by-product meal from 28 to 42 d of age. *British Poultry Science*, 52(2), 238-244. <https://doi.org/10.1080/00071668.2011.561282>

- Duo, H., Zunjare, R. U., Mishra, S. J., Muthusamy, V., Thambiyannan, S., Kumar, S., Kasana, R. K., Gopinath, I., Sharma, G., & Chhabra, R. (2024). Genetic analysis on composition of sulfur-containing amino acids: Methionine and cysteine in subtropical maize. *Journal of Food Composition and Analysis*, 136, Article 106774. <https://doi.org/10.1016/j.jfca.2024.106774>
- Fijalovych, L., Kyrlyliv, Y. I., Barylo, B., Paskevych, G., Petryshak, O., & Deneha, U. (2022). Effectiveness of different protein sources and lysine levels in diets for broiler chickens. *Scientific Messenger of LNU of Veterinary Medicine and Biotechnologies. Series: Agricultural Sciences*, 24(97), 99-105. <https://doi.org/10.32718/nvlvet-a9717>
- Hagan, N., & Higgins, T. (2024). Enhancing the nutritive value of seeds by genetic engineering. In R. L. Bhatia (Ed.), *Handbook of seed science and technology* (pp. 171-193). CRC Press. <https://doi.org/10.1201/9781003578369-8>
- Han, M., Zhang, C., Suglo, P., Sun, S., Wang, M., & Su, T. (2021). L-Aspartate: An essential metabolite for plant growth and stress acclimation. *Molecules*, 26(7), Article 1887. <https://doi.org/10.3390/molecules26071887>
- Hasan, M. M. (2024). Towards programming-based synthetic genetic circuit enabled high-lysine maize. *Current Plant Biology*, 39, Article 100355. <https://doi.org/10.1016/j.cpb.2024.100355>
- Hasan, M. M., & Rima, R. (2021). Genetic engineering to improve essential and conditionally essential amino acids in maize: Transporter engineering as a reference. *Transgenic Research*, 30(2), 207-220. <https://doi.org/10.1007/s11248-021-00235-0>
- Hu, X., Kong, L., Xiao, C., Zhu, Q., & Song, Z. (2021). The AMPK-mTOR signaling pathway is involved in regulation of food intake in the hypothalamus of stressed chickens. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 258, Article 110979. <https://doi.org/10.1016/j.cbpa.2021.110979>
- Jankowski, J., Kubińska, M., & Zduńczyk, Z. (2014). Nutritional and immunomodulatory function of methionine in poultry diets - a review. *Annals of Animal Science*, 14(1), 17-32. <https://doi.org/10.2478/aoas-2013-0081>
- Khwatenge, C. N., Kimathi, B. M., Taylor-Bowden, T., & Nahashon, S. N. (2020). Expression of lysine-mediated neuropeptide hormones controlling satiety and appetite in broiler chickens. *Poultry Science*, 99(3), 1409-1420. <https://doi.org/10.1016/j.psj.2019.10.053>
- Klann, K., Tascher, G., & Münch, C. (2020). Functional translational proteomics reveal converging and dose-dependent regulation by mTORC1 and eIF2alpha. *Molecular Cell*, 77(4), 913-925. <https://doi.org/10.1016/j.molcel.2019.11.010>
- Klünemann, M., Romero, L., Acman, M., Milfort, M., Fuller, A., Rekaya, R., Aggrey, S., Payling, L., & Lemme, A. (2024). Multitissue transcriptomics demonstrates the systemic physiology of methionine deficiency in broiler chickens. *Animal*, 18(5), Article 101143. <https://doi.org/10.1016/j.animal.2024.101143>
- Kwak, M.-J., Kang, A., Eor, J., Ryu, S., Choi, Y., Heo, J. M., Song, M., Kim, J. N., Kim, H.-J., & Kim, Y. (2024). Dietary L-methionine modulates the gut microbiota and improves the expression of tight junctions in an *in vitro* model of the chicken gastrointestinal tract. *Animal Microbiome*, 6(1), Article 14. <https://doi.org/10.1186/s42523-024-00303-w>

- Lee, C. Y., Song, A. A., Loh, T. C., & Abdul Rahim, R. (2020). Effects of lysine and methionine in a low crude protein diet on the growth performance and gene expression of immunity genes in broilers. *Poultry Science*, 99(6), 2916-2925. <https://doi.org/10.1016/j.psj.2020.03.013>
- Linh, N., Vui, N., Guntoro, B., & Qui, N. (2021). The effects of dietary methionine during 5-14 weeks of age on growth performance and carcass traits of chickens. *Journal of Animal Health and Production*, 9(2), 193-197. <https://doi.org/10.17582/journal.jahp/2021/9.2.193.197>
- Linh, N. T., Guntoro, B., & Qui, N. H. (2021). Immunomodulatory, behavioural, and nutritional response of tryptophan application on poultry. *Veterinary World*, 14(8), 2244-2250, Article 34. <https://doi.org/10.14202/vetworld.2021.2244-2250>
- Lisnahan, C. V., & Nahak, O. R. (2020). Growth performance and small intestinal morphology of native chickens after feed supplementation with tryptophan and threonine during the starter phase. *Veterinary World*, 13(12), 2765-2771, Article 23. <https://doi.org/10.14202/vetworld.2020.2765-2771>
- Macelline, S. P., Chrystal, P. V., McQuade, L. R., McInerney, B. V., Kim, Y., Bao, Y., Selle, P. H., & Liu, S. Y. (2021). Graded methionine dietary inclusions influence growth performance and apparent ileal amino acid digestibility coefficients and disappearance rates in broiler chickens. *Animal Nutrition*, 8, 160-169. <https://doi.org/10.1016/j.aninu.2021.06.017>
- Maqbool, M. A., Beshir Issa, A., & Khokhar, E. S. (2021). Quality protein maize (QPM): Importance, genetics, timeline of different events, breeding strategies and varietal adoption. *Plant Breeding*, 140(3), 375-399. <https://doi.org/10.1111/pbr.12923>
- Maqsood, M. A., Khan, E. U., Qaisrani, S. N., Rashid, M. A., Shaheen, M. S., Nazir, A., Talib, H., & Ahmad, S. (2022). Interactive effect of amino acids balanced at ideal lysine ratio and exogenous protease supplemented to low CP diet on growth performance, carcass traits, gut morphology, and serum metabolites in broiler chicken. *Tropical Animal Health and Production*, 54, Article 186. <https://doi.org/10.1007/s11250-022-03184-w>
- Maynard, C. W., Kidd, M. T., Chrystal, P. V., McQuade, L. R., McInerney, B. V., Selle, P. H., & Liu, S. Y. (2022). Assessment of limiting dietary amino acids in broiler chickens offered reduced crude protein diets. *Animal Nutrition*, 10, 1-11. <https://doi.org/10.1016/j.aninu.2021.11.010>
- Mund, M. D., Riaz, M., Mirza, M. A., Rahman, Z. -U., Mahmood, T., Ahmad, F., & Ammar, A. (2020). Effect of dietary tryptophan supplementation on growth performance, immune response and antioxidant status of broiler chickens from 7 to 21 days. *Veterinary Medicine and Science*, 6(1), 48-53. <https://doi.org/10.1002/vms3.195>
- Nagesh, C., Prashat G, R., Goswami, S., Bharadwaj, C., Praveen, S., Ramesh, S., & Vinutha, T. (2024). Sulfate transport and metabolism: Strategies to improve the seed protein quality. *Molecular Biology Reports*, 51, Article 242. <https://doi.org/10.1007/s11033-023-09166-x>
- Naveen, A. K., & Sontakke, M. (2024). A review on regulatory aspects, challenges and public perception in acceptance of genetically modified foods. *Food Science and Biotechnology*, 33(4), 791-804. <https://doi.org/10.1007/s10068-023-01481-0>
- Pope, M., Boyd, R. D., Holzgraefe, D., Monaco, H., & Sifri, M. (2024). Economic value and environmental impact of soybean meal in poultry and swine diets. *Journal of Applied Poultry Research*, 33(3), Article 100449. <https://doi.org/10.1016/j.japr.2024.100449>

- Rajah Kumar, M., Amankwaa, A. O., Razali, N. S., Mohamad, N. E., Khalid, M., Abdullah, J. O., Yeap, S. K., Masarudin, M. J., & Alitheen, N. B. (2025). Preliminary evaluation of the gut microbiota modulatory potential of Malaysian kefir water in ageing mice. *Foods*, 14(22), Article 3851. <https://doi.org/10.3390/foods14223851>
- Ramadan, N., Shaib, H., & Farran, M. (2021). Nutritional and immunopotentiating function of methionine for poultry. *International Journal of Plant, Animal and Environmental Sciences*, 11(3), 363-390. <https://doi.org/10.26502/ijpaes.202111>
- Sheoran, S., Kumar, S., Ramtekey, V., Kar, P., Meena, R. S., & Jangir, C. K. (2022). Current status and potential of biofortification to enhance crop nutritional quality: An overview. *Sustainability*, 14(6), Article 3301. <https://doi.org/10.3390/su14063301>
- Song, B., Fu, M., He, F., Zhao, H., Wang, Y., Nie, Q., & Wu, B. (2021). Methionine deficiency affects liver and kidney health, oxidative stress, and ileum mucosal immunity in broilers. *Frontiers in Veterinary Science*, 8, Article 722567. <https://doi.org/10.3389/fvets.2021.722567>
- Taylor-Bowden, T., Bhogoju, S., Khwatenge, C. N., & Nahashon, S. N. (2024). The impact of essential amino acids on the gut microbiota of broiler chickens. *Microorganisms*, 12(4), Article 693. <https://doi.org/10.3390/microorganisms12040693>
- Te Pas, M. F., Veldkamp, T., de Haas, Y., Bannink, A., & Ellen, E. D. (2021). Adaptation of livestock to new diets using feed components without competition with human edible protein sources - a review of the possibilities and recommendations. *Animals*, 11(8), Article 2293. <https://doi.org/10.3390/ani11082293>
- Tien Lea, D., Duc Chua, H., & Quynh Lea, N. (2016). Improving nutritional quality of plant proteins through genetic engineering. *Current Genomics*, 17(3), 220-229. <https://doi.org/10.2174/1389202917666160202215934>
- Tian, D., Guo, R., Li, Y., Chen, P., Zi, B., Wang, J., Liu, R., Min, Y., Wang, Z., & Niu, Z. (2019). Effects of lysine deficiency or excess on growth and the expression of lipid metabolism genes in slow-growing broilers. *Poultry Science*, 98(7), 2927-2932. <https://doi.org/10.3382/ps/pez041>
- Tran, D. H., Schonewille, J. T., Pukkung, C., & Khempaka, S. (2021). Growth performance and accretion of selected amino acids in response to three levels of dietary lysine fed to fast- and slow-growing broilers. *Poultry Science*, 100(4), Article 100998. <https://doi.org/10.1016/j.psj.2021.01.021>
- Tukuli, A. R. (2022). *Increasing methionine content of soybean using CRISPR/Cas9 and developing machine learning predictive models* [Doctoral dissertation, University of Missouri-Columbia].
- Vastolo, A., Serrapica, F., Cavallini, D., Fusaro, I., Atzori, A. S., & Todaro, M. (2024). Alternative and novel livestock feed: Reducing environmental impact. *Frontiers in Veterinary Science*, 11, Article 1441905. <https://doi.org/10.3389/fvets.2024.1441905>
- Vazquez-Anon, M., Gonzalez-Esquerria, R., Saleh, E., Hampton, T., Ritcher, S., Firman, J., & Knight, C. (2006). Evidence for 2-hydroxy-4-(methylthio)butanoic acid and DL-methionine having different dose responses in growing broilers. *Poultry Science*, 85(8), 1409-1420. <https://doi.org/10.1093/ps/85.8.1409>
- Wang, W., & Bin, C. (2024). Investigating the molecular mechanisms of lysine's effect on feed intake in chicks through the hypothalamus using RNAseq and metabolomics. *Indian Journal of Animal Research*, 58(12), 1954-1962. <https://doi.org/10.18805/IJAR.BF-1844>

- Wu, P., Yuan, Y., Ma, Z., Zhang, K., Deng, L., Ren, H., Yang, W., & Wang, W. (2024). Comparative transcriptome profiling reveals the mechanism of increasing lysine and tryptophan content through pyramiding *opaque2*, *opaque16* and *waxy1* genes in maize. *Breeding Science*, 74(4), 311-323. <https://doi.org/10.1270/jsbbs.23051>
- Yang, Q., Zhao, D., & Liu, Q. (2020). Connections between amino acid metabolisms in plants: Lysine as an example. *Frontiers in Plant Science*, 11, Article 928. <https://doi.org/10.3389/fpls.2020.00928>
- Yoon, J. H., An, S. H., Kim, Y., Kim, H.-J., & Kong, C. (2024). Growth performance of broiler chickens fed diets containing granulated L-methionine compared with DL-methionine. *Journal of Animal Science and Technology*, 66(4), 743-755. <https://doi.org/10.5187/jast.2024.e49>
- Zafar, S., & Jianlong, X. (2023). Recent advances to enhance nutritional quality of rice. *Rice Science*, 30(6), 523-536. <https://doi.org/10.1016/j.rsci.2023.05.004>
- Zhang, T., Li, J., Hu, J., Chen, Y., Fu, X., Zhao, W., Tian, J., Yang, L., & Zhang, J. (2024). Identification of key differentially methylated genes regulating muscle development in chickens: Insights from Jingyuan breed. *Poultry Science*, 103(12), Article 104292. <https://doi.org/10.1016/j.psj.2024.104292>

Review Article

Bulking Agents and Composting Methods Influence the Physicochemical Quality of Aerobic Chicken Manure Compost: A Comprehensive Review

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ABSTRACT

The aerobic composting of chicken manure (CM) has developed through continuous research and experimentation to optimise compost quality while concurrently improving environmental safety and public perception. Successful composting often requires a suitable carbon-to-nitrogen ratio (C/N) in a balanced combination of feedstock and bulking agent for effective microbial degradation. Despite numerous trials using different bulking agents, a comprehensive review of the aspect of practicality and effectiveness on physicochemical quality parameters remains unclear in current studies. Hence, this study investigates the influence of distinct bulking agent categories and composting methods of CM on key physicochemical parameters, which include C/N, pH, electrical conductivity (EC), nitrogen, phosphorus (P), potassium (K), bulk density (BD), moisture content (MC) and composting duration (CD). Plant-derived materials and livestock manure serve as the primary bulking agents, which significantly affect C/N, EC, K, BD and CD. The windrow method

provided better retention of P and K contents, while the in-vessel method improved C/N, EC, BD, MC and CD. These reviews highlight the importance of selecting suitable bulking agents and composting methods, as their interactions directly influence CM compost quality.

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INTRODUCTION

Chicken manure (CM) is recognised as one of the most produced livestock waste globally, primarily due to its intensive production worldwide to meet the demand for animal protein (Food and Agriculture Organisation [FAO], 2023). Farmers have historically favoured CM as a soil amendment over other livestock manures due to its well-known high concentration of macronutrients, which has proven to yield better agricultural harvests (Oagile & Namasiku, 2010; Warman, 1986). Aerobic composting, which involves the breakdown of organic matter in the presence of oxygen, has emerged as a popular treatment option due to its cost-effectiveness, requiring less mechanisation, and applicability for both urban and rural settings (Congilosi & Aga, 2021). Feedstock refers to the raw organic materials in the compost pile, which significantly influence both the nature of the compost and the composting process itself, often more than any other factors (Rynk et al., 2022). Additional feedstock introduced during composting, besides the primary feedstock, is also referred to interchangeably as a bulking agent (Manish et al., 2013). In this review, CM is the primary feedstock utilised, which can be composted solely or co-composted with a bulking agent. The benefits of bulking agent include the conditioning of initial composting composition, enhancing density, providing inter-particle gaps and air space, regulating moisture levels and serving as a carbon source for microorganisms (Chang et al., 2022; Chowdhury et al., 2014; Oshins et al., 2022; Santos et al., 2016; Yang et al., 2013).

The type and amount of bulking agent utilised in composting could largely influence the compost maturation time, emissions of gases, and overall compost quality (Li et al., 2018; Zhu, 2007). Common examples of bulking agents include wood chips, wood shavings, sawdust, dry leaves, food waste, shredded landscape waste, shredded paper, shredded cardboard, and animal bedding (Manish et al., 2013). Although these bulking agents are often considered waste materials, discarding them without fully utilising their abundant nutrient content would be inefficient. Composting not only mitigates pollution but also effectively reutilises the residual energy and nutrient resources (Grisey & Aleya, 2015; Paredes et al., 1996).

Moreover, the daily mass production of waste has given rise to several environmental challenges. Leaching of excess nutrients into waterways, which leads to eutrophication and the production of greenhouse gases caused by large volumes of waste storage, is among the factors that contribute to the contentious issue of climate change (Fernando, 2019; Kaza et al., 2018; Mehta et al., 2015). Subsequently, an ideal feedstock combination is critical to ensure optimal composting procedures in producing high-quality and sustainable compost products. Chai et al. (2013) and Rynk et al. (2022) have emphasised that farmers and researchers have been experimenting with various compositions and bulking agents when composting chicken manure to achieve the best outcomes. The methods applied during CM composting also play a vital role in determining the final quality of the compost.

Popular methods consist of static pile, vermicomposting, bokashi, windrow and in-vessel methods. The choice of composting method is influenced by various factors, including the type of feedstock, operational scale, financial considerations, regulatory requirements, and the degree of community sensitivity (Oshins et al., 2022). Since the most widely adopted methods are in-vessel and windrow composting systems (Kim et al., 2008; Manyapu et al., 2017; Zhou et al., 2022), this review will particularly focus on analysing the impact of in-vessel and windrow composting systems on compost physicochemical parameters.

The objectives of this review are to (a) analyse the effect of different bulking agents on compost physicochemical parameters (carbon-to-nitrogen ratio, pH, electrical conductivity, nitrogen, phosphorus, potassium, bulk density, moisture content), and composting duration; (b) investigates the effects of composting methods (in-vessel and windrow) on physicochemical parameters and composting duration; and (c) identify significant correlations among the physicochemical parameters during aerobic composting of CM. Due to the wide variations of bulking agents used in CM composting, this paper aims to unravel the complex relationship between bulking agents, composting methods, and the physicochemical quality of the resulting compost.

MATERIALS AND METHODS

Literature Search

The literature search utilised various online research databases, including Science Direct, Elsevier, MDPI, PLOS One, ResearchGate, Wiley Online Library, Scopus, Taylor & Francis, Springer, and Frontiers in Microbiology were used in the literature search. A total of 79 research articles which focus on assessing physicochemical parameters during aerobic composting of CM were selected in this review. The chosen studies were mostly published regarding either in-vessel or windrow composting systems between 2014 to 2023. Hence, this review covered 125 distinct compost samples. The methodological approach of this review draws from a broad and reputable range of article databases, a focused ten-year window, and a substantial set of 125 distinct compost samples that lend strong credibility and robustness to this comprehensive review.

Data Analysis

Microsoft Excel was implemented in this review for data management and descriptive analysis. Data collected from different studies were standardised into consistent units to facilitate comparison. The dataset was then classified based on authors, publication year, continent, composting method and duration. Comparative analysis was conducted on physicochemical parameters like carbon-to-nitrogen ratio (C/N), pH, electrical conductivity (EC), nitrogen (N), phosphorus (P), potassium (K), bulk density (BD), and moisture content (MC), along with composting duration (CD).

OVERVIEW OF THE SELECTED LITERATURE

The review compiled global studies on the physicochemical quality parameters of aerobic composting of CM across nine continents, which were Far East Asia, Southeast Asia, South Asia, North America, South America, Africa, Australia, Europe and the Middle East. The regional data distribution based on individual treatments collected from the selected studies was illustrated in Figure 1.

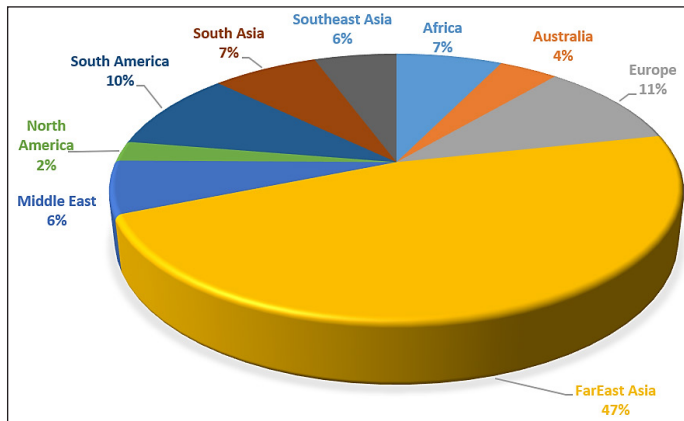


Figure 1. Regions explained with the number of data, Southeast Asia (n = 7), South Asia (n = 9), Far-east Asia (n = 59), North America (n = 3), South America (n = 12), Africa (n = 9), Australia (n = 5), Europe (n = 13), Middle East (n = 8), all regions (n = 125)

Fareast Asia contributes almost half (47%) of the total studies conducted on physicochemical quality parameters of CM composting, primarily from China. This shows their contribution in managing extensive waste production and developing better compost quality. Furthermore, China ranks as the third-largest global chicken producer, after the United States and Brazil (Shahbandeh, 2023; Wen et al., 2019). Other continents contributed approximately equal percentages of studies to this review, with the lowest percentage of 2% from North America.

OPTIMAL PHYSICOCHEMICAL COMPOSTING CONDITIONS

The optimal composting conditions outlined in this section serve as benchmarks for evaluating compost quality parameters and providing references for subsequent discussions. The key physicochemical parameters reviewed in this study were C/N, pH, EC, N, P, K, BD and MC. C/N ratio determines the stability and maturity of compost (Oshins et al., 2022). Carbon serves as the primary energy source for microbial growth, while N is necessary for microbial development and reproduction through protein synthesis (Sweeten, 2008). The ideal final C/N falls within the range of 20:1 to 30:1, as microbes require 20 to 30 parts of carbon per unit of N for optimal microbial activity (Estévez-Schwarz et al., 2012; Guo et

al., 2022 ; Ogunwande, 2008; Rynk et al., 1992). Compost pH reflected the hydrogen ion activity, which was influenced by mixing and concentrations of cations and anions in the composting system (Butterly et al., 2013; Smith & Doran, 2015). Most composts tend to maintain a neutral to slightly alkaline pH, with the optimal pH range between 6.5 and 8.0 (Ho et al., 2022; Oshins et al., 2022).

Compost salinity was assessed by measuring the EC of its water extract (Thompson et al., 2002). High levels of salt concentrations negatively affect plant tissues and reduce their root capacity for water adoption from the soil compost (Gondek et al., 2020). EC values lower than 4 dS m⁻¹ are generally considered ideal and non-phytotoxic (Awasthi et al., 2016; Manyapu et al., 2017). Higher macronutrient concentrations are often assumed to improve soil fertility and crop performance. However, recommended compost quality standards suggest a range of 1.0 - 3.5% for N, <1.0% for P, and 1.0 -3.0% for K (Nyi et al., 2017; Heyman et al., 2019). BD represents the ratio of a material's mass to its occupied volume, which is considered the key indicator of aeration within the composting mix (Calisti et al., 2020 ; Oshins et al., 2022). Practically, BD ranging from 400-600 kg/m³ is advisable (Oshins et al., 2022) to enhance permeability, adequate air space, oxygen utilisation efficiency and heat transfer. Due to moisture-filled pores or denser piles, excessively high BD occurred and led to a decrease in porosity and limited aeration. In contrast, extremely low BD can cause unnecessary substrate aeration and reduced water availability (Azim et al., 2018).

Moisture content was widely recognised as the second most important factor in influencing the rate of decomposition, after the C/N ratio (Choi, 1999; Sharma & Reynnells, 2018). Effective composting requires careful monitoring of moisture or humidity levels, ideally maintaining them between 40-60% by weight (Singh & Kalamdhad, 2015 ; Oshins et al., 2022). Microorganisms require water for transporting nutrients and energy elements through their cell membrane; thus, regulating moisture levels becomes critical for successful composting. Inadequate moisture levels can restrict the growth and survival of microorganisms within the compost (Sharma & Reynnells, 2016). Nevertheless, it is important to acknowledge that an ideal compost is a combination of multiple factors highly influenced by the environment and ambient conditions, and there is no universally "ideal" compost; rather, there are several types, each designed for specific purposes.

Composting consists of three commonly identified phases: the mesophilic (moderate-temperature phase), which lasts for a couple of days, the thermophilic (high-temperature phase), which lasts for a few days to a few weeks, and the long maturation (cooling) phase (Pace et al., 1995; Wong et al., 2017). The composting process begins as soon as the raw materials are combined. In the initial stage, microbes rapidly consume oxygen and easily degradable components of the source materials. As active composting slows down, the temperature gradually decreases until the compost reaches ambient air temperature. The active composting phase is followed by a curing phase, where the components will break

down progressively. This continues until the final degradable raw materials are utilised by the remaining microbes. At this stage, the compost becomes less odorous, free of pathogens, reduced in volume, easier to manage, and relatively stable (Wang et al., 2016; Wong et al., 2017). The general perception is that composting phases are time-consuming; shorter compost maturation durations are preferred.

DIFFERENT BULKING AGENTS USED IN CHICKEN MANURE COMPOSTING

Most of the bulking agents were selected due to their production as by-products and being commonly perceived as agricultural wastes. Co-composting them with CM concurrently addresses the waste management issue for both parties. Another general reason is that these incorporated materials help to compensate for the shortcomings of CM, thereby enhancing various aspects of the composting process, including structural, biological, and chemical parameters, ultimately improving the quality of the final compost. The effect of various bulking agent categories used in CM composting will be investigated by analysing individual treatments ($n = 125$) in studies conducted between 2014 and 2023. They are classified into different categories, including plant-derived, livestock manure, organic waste, animal by-product, rock, industrial by-product, animal, and bio-surfactants (Table 1). Discussion will focus on bulking agent categories with more than three treatments ($n > 3$), namely plant-derived material and livestock manure.

Plant-Derived Material as Bulking Agent

This review has found that bulking agents derived from plants are the preferred choice for composting CM. This preference may be attributed to the high-carbon compounds found in plant wastes, constituting up to 30% of total organic carbon (Azim et al., 2018; Vallini et al., 1993). Bulking agents rich in carbon compounds are essential to attain optimal C/N of 20:1 -30:1, especially considering the high N content in CM (Nahm, 2003). This balance enhances optimal microbial activity during the composting process, making the addition of plant-derived materials as bulking agents crucial for this purpose. Additionally, the lignocellulosic properties in plants also contribute to the structural degradation and biochemical enhancement of the composting process and the resulting compost product (Hubbe et al., 2010). Watermelon waste serves as an effective bulking agent, significantly enhancing the organic matter, N, K, dry matter, moisture content, bulk density, and pH on the final compost (Nurin et al., 2024).

Livestock Manure Bulking Agent

Co-composting with two or more distinct livestock manures is a popular disposal method aimed at optimising the priming effect and antagonistic reactions among the vast

Table 1
Categorisation of bulking agent types

Bulking Agent Category and Definition		List of Materials
Plant-derived is coming entirely from plants, plant-derived materials, or plant by-products.		(1) sugarcane waste, (2) rice husk, (3) gamal leaves, (4) agricultural lime, (5) molasses, (6) <i>Cattha edulis</i> leaf wastes, (7) farm wastes, (8) wheat straw, (9) rice straw, (10) mushroom dreg, (11) rice bran, (12) banana peels, (13) sawdust, (14) rice hull, (15) biochar, (16) barley straw, (17) <i>Caragana microphylla</i> straw, (18) rice husk biochar, (19) bagasse, (20) <i>Eucalyptus</i> sp. sawdust, (21) coir pith, (22) vegetable waste, (23) coffee husk, (24) pod husk mixed with topsoil, (25) pine sawdust, (26) macadamia nutshell biochar, (27) hardwood shavings biochar, (28) olive leaves and pruning, (29) alperujo cereal straw, (30) exhausted grape marc, (31) gorse forest shrub, (32) bamboo charcoal, (33) tomato stalk, (34) peat bog, (35) fruit and vegetable residue, (36) oil palm empty fruit bunch biochar, (37) corn stalk, (38) straw pellets, (39) green algae, (40) olive mill wastewaters, (41) olive pomace, (42) corn straw, (43) rapeseed meal, (44) green waste, (45) greenhouse tomato
Animal Manure refers to solid, semisolid, and liquid waste generated by livestock animals or their derived materials		(1) swine manure, (2) poultry litter, (3) dairy manure, (4) chicken litter biochar, (5) horse manure compost
Organic Waste refers to organic materials derived from living organisms or their metabolic processes		(1) market organic waste, (2) household organic waste, (3) sewage sludge
Animal by-product refers to materials of animal origin that are inedible		(1) urea, (2) beef rumen content, (3) meat meal
Rocks refer to a solid collection of mineral grains that possess a high amount of carbon		(1) pumice, (2) chabazite, (3) dolomitic limestone, (4) zeolite, (5) diatomite, (6) bentonite
Industrial by-product refers to residual material generated from an industrial or commercial operation that is not the primary product of that operation		(1) sugar filter cake waste
Animal refers to bulking agents coming entirely from animal and animal-derived materials		(1) black soldier fly larvae (BSFL), (2) larva inocula, (3) <i>B. subtilis</i> BSF-CL, (4) dead birds
Bio-surfactants are produced by bacteria, yeasts, and filamentous fungi, which have low toxicity, high biodegradability, and remain active at extreme pH and salinity		(1) Rhamnolipid, (2) Tween-80

microorganisms, nutrient compositions, and physicochemical conditions derived from the combination of multiple types of manure in the compost mix (Huang et al., 2017; Neher et al., 2020). Nurin et al. (2025) reported that application of sawdust as bulking agent improved the overall quality in co-composting of CM with horse manure. This approach helps in enhancing organic matter degradation and elevating nutrient conservation, thereby improving compost quality and mitigating potential environmental effects associated with the mass production of livestock manures (Hwang et al., 2020; Song et al., 2014).

This review identified several livestock manures previously utilised as bulking agents in aerobic composting of CM, including swine manure, chicken litter, dairy manure, chicken litter biochar, and horse manure compost (Table 1). Co-composting chicken with swine manures was found to significantly increase the quantity of xylan-degrading bacteria and reduce the number of human pathogens (Song et al., 2014). However, Hwang et al. (2020) revealed that co-composting CM with swine manure released 4.1 times more NH_3 emissions compared with dealing with CM alone. Co-composting CM with dairy manure increases lignin, cellulose, and hemicellulose degradation, leading to higher organic mass reduction by the end of the composting period (Rehman et al., 2017). Since chicken, swine, and cattle manures are high in N, their incorporation often results in very low C/N and significant N loss through ammonia volatilisation during composting (Biala et al., 2016).

The utilisation of manure biochar, produced through pyrolysis, is increasingly common as a bulking agent during composting. The application brings several advantages, including providing a high carbon source contributing to optimal microbial activity, being lighter and thus more cost-effective for transport, emitting less odour, and offering beneficial soil amendment properties. Moreover, it has been proven to enhance the composting process by promoting the nitrification process, reducing methane emissions, and eliminating pathogens (Agyarko-Mintah et al., 2017; nase et al., 2014). Liu et al. (2018) conducted a study on co-composting CM with mature horse compost. They reported that sodium content dilution and microbial activity were enhanced, resulting in optimal compost temperature and better maturity indices compared to composting CM with vegetable or food waste. Livestock manure as a bulking agent has long been applied in CM composting, and its incorporation has brought various benefits to the entire composting process.

EFFECTS OF DIFFERENT BULKING AGENT CATEGORIES ON COMPOST PHYSICOCHEMICAL PARAMETERS

CM was composted without bulking agent (WO) ($n = 20$) and co-composted with the following bulking agent categories; plant-derived materials ($n = 79$), livestock manure ($n = 5$), livestock manure and plant-derived ($n = 5$), animal ($n = 2$), organic waste ($n = 2$), rock ($n = 2$), animal by-product ($n = 1$), animal and plant-derived ($n = 1$), industrial by-product ($n = 1$), plant-derived and industrial by-product ($n = 1$), plant-derived and

bio-surfactants ($n = 2$), rock and plant-derived ($n = 2$), rock and animal ($n = 1$), animal by-product and plant-derived ($n = 1$). This review focuses on the effect of bulking agent categories on compost physicochemical parameters with more than three treatments ($n > 3$), which include CM composted without a bulking agent (CM-WO), CM co-composted with plant-derived materials (CM-P), CM co-composted with animal manure (CM-M), and CM co-composted with animal manure and plant-derived materials (CM-MP). Summary statistics of the physicochemical parameters of the final CM compost; comparisons between bulking agent categories are presented in Table 2.

Carbon-to-Nitrogen Ratio

C/N is a crucial parameter in composting, signifying the balance between carbon-rich and nitrogen-rich materials for optimal microbial activity (Khan et al., 2014). The C/N for each bulking agent category fall below the ideal range of 20:1 to 30:1. CM-MP shows the highest average value (19.6), followed by CM-P (17.8), CM-M (14.6), and CM-WO (12.9). CM-MP nearly approaches the optimal C/N range. This review highlights that the incorporation of CM solely with plant-derived materials (CM-P) or livestock manures (CM-M) does not improve the final C/N to the same extent as the combination of both materials does. This signifies limitations of each bulking agent category, and the application of both materials as bulking agents during the composting of CM is recommended to achieve better C/N in the final compost. Comparatively, Liu et al. (2018) found that co-composting CM with livestock manure was more efficient in degradation rate as compared to using plant-derived material as a bulking agent. Additionally, CM-WO has the lowest C/N, emphasising that composting CM without any bulking agent is not advisable. The imbalance in organic matter composition resulting from the absence of a bulking agent will consequently lead to immature C/N in the final compost.

pH and Electrical Conductivity

Compost pH plays a significant role in plant growth as it impacts various soil parameters such as nutrient availability, heavy metals toxicity, soil microbial composition, and activity, as well as cation exchange capacity (Oshins et al., 2022; Zhang et al., 2021). In this review, the average pH values for each bulking agent category were found to be within an acceptable range when compared with the ideal range of 6.5 -8.0 (Ho et al., 2022; Oshins et al., 2022). The results span from CM-WO (7.7) to CM-P (8.1), showing a very narrow variance between the bulking agent categories, suggesting that each of them did not have a significant negative effect on the final pH value in CM composting.

The electrical conductivity value reflects the total concentration of dissolved salts and ions in compost, directly impacting nutrient availability, microbial activity, and overall compost quality. As shown in Table 2, there is a considerable variation in EC values, ranging

Table 2
Summary statistics of the physicochemical parameters of final chicken manure compost; comparisons between bulking agent categories

Bulking Agent Category		Physicochemical Parameter									
		C/N	pH	EC (dS m ⁻¹)	N (%)	P (%)	K (%)	PS (cm)	BD (kg/m ³)	MC (%)	CD (day)
Without a Bulking Agent (CM-WO)	Mean	12.9 ± 6.8 (n = 20)	7.7 ± 0.8 (n = 19)	6.6 ± 9.1 (n = 9)	2.4 ± 1.2 (n = 20)	1.4 ± 0.7 (n = 15)	2.3 ± 1.5 (n = 14)	0.4 ± 0.1 (n = 2)	521.8 ± 223.7 (n = 5)	42.5 ± 24.8 (n = 11)	98.7 ± 90.7 (n = 15)
	± SD										
	Min	6.3	6.4	0.2	0.3	0.00004	0.5	0.3	250	15	9
	Max	37.2	9.2	29.1	4.3	2.8	5.9	0.5	810	88.4	365
Plant-derived (CM-P)	Mean	17.8 ± 7.8 (n = 73)	8.1 ± 0.9 (n = 71)	3.8 ± 2.9 (n = 41)	1.8 ± 1 (n = 48)	1.5 ± 2.1 (n = 36)	1.8 ± 1.9 (n = 34)	0.8 ± 0.5 (n = 5)	401.3 ± 127.9 (n = 8)	47.8 ± 15.8 (n = 43)	80.7 ± 81 (n = 74)
	± SD										
	Min	2.2	6	0.003	0.1	0.0002	0.001	0.03	260	11.9	0.9
	Max	43.1	11	11.7	5.0	11.3	8.5	1.5	570	67.6	600
Animal Manure (CM-M)	Mean	14.6 ± 7.7 (n = 5)	7.8 ± 1 (n = 5)	2.0 ± 2.5 (n = 2)	2.3 ± 1.3 (n = 5)	1.2 ± 0.2 (n = 4)	1.9 ± 0.9 (n = 3)	-	-	56.6 ± 21.5 (n = 5)	65.3 ± 39.5 (n = 3)
	± SD										
	Min	9.8	6.7	0.2	0.2	1.0	0.9	-	-	34.0	20
	Max	28	9.2	3.8	3.7	1.4	2.7	-	-	88.9	92
Animal Manure + Plant-derived (CM-MP)	Mean	19.6 ± 9.8 (n = 5)	7.9 ± 0.8 (n = 5)	5.2 ± 3.5 (n = 5)	2.1 ± 0.7 (n = 4)	11.8 ± 2.2 (n = 2)	± 0.1 (n = 4)	-	-	52.5 ± 10.2 (n = 3)	81 ± 45 (n = 5)
	± SD										
	Min	10.2	7.3	3.0	1.5	10.3	0.002	-	-	40.9	30
	Max	35.7	9.0	11.4	3.0	13.4	0.03	-	-	60	126

Note. CM-WO, chicken manure composted without bulking agent; CM-P, chicken manure co-composted with plant-derived materials; CM-M, chicken manure co-composted with livestock manure; CM-MP, chicken manure co-composted with livestock manure and plant-derived materials; SD, standard deviation; C/N, carbon-to-nitrogen ratio; EC, electrical conductivity; N, nitrogen; P, phosphorus; K, potassium; PS, particle size; BD, bulk density; MC, moisture content; CD, composting duration. Number of data reviewed for this analysis, n = 101

from the lowest value in CM-M (2.0 dS m^{-1}), followed by CM-P (3.8 dS m^{-1}), CM-MP (5.2 dS m^{-1}), to the highest in CM-WO (6.6 dS m^{-1}). Both CM-M and CM-P fall within the optimal EC range below 4 dS m^{-1} , indicating an adequate concentration of dissolved salts that creates optimal conditions for plant nutrient uptake efficiency (Gondek et al., 2020). The high EC value in CM-MP may be attributed to the microbial dynamics and high nutrient content resulting from the combination of both plant-derived material and livestock manure (Lauber et al., 2009; Zhang et al., 2018). The extremely high EC value observed in CM-WO could be attributed to its low C/N, which accelerates mineralisation, producing more N compounds such as ammonium, nitrates, and organic nitrogen. These organic compounds contain electrolytes that contribute to the high EC value (Carmo et al., 2016). This indicates the importance of selecting compatible feedstock and bulking agents to maintain suitable pH and EC levels as well as minimising the risk of phytotoxic effects on plant growth and productivity.

Macronutrient Contents

This review focuses on the primary macronutrients essential for optimal compost quality, which were N, P and K (Sánchez et al., 2017). Previous studies have highlighted a significant correlation between feedstock composition and the rate of nutrient mineralisation (Silva et al., 2014; Wang et al., 2019). Hence, this review determined the variations in macronutrient contents across different types of bulking agent, indicating their effects on nutrient dynamics of composting. The average N values in increasing order were observed in CM-P (1.8%), CM-MP (2.1%), CM-M (2.3%) and CM-WO (2.4%). The lowest N content recorded in CM-P is likely due to the high carbon content presented in plant-derived materials. This creates a higher initial C/N ratio, which can reduce N mineralisation during the composting process and consequently the final concentration (Oshins et al., 2022). However, no significant difference in N content was found among the other bulking agent categories. The highest N content in CM-WO may be owing to the high net mineralisation resulting from a low initial C/N ratio (Eckhardt et al., 2018). In addition, all bulking agent categories exhibited N levels within the acceptable range defined by established compost quality standards recommended by Nyi et al. (2017) and Heyman et al. (2019).

The contrasting trends observed in P and K contents among the various bulking agent categories emphasise their contradictory impacts on nutrient composition. CM is well-known for its highest P content among other organic wastes, requiring an optimised mode of composting to maximise P reutilisation (Wei et al., 2015). The variation of P between the bulking agent categories is very narrow, ranging from 1.2% - 1.5%. This suggests that every category retains a relatively high concentration of P in the compost, implying that feedstock composition does not significantly affect P content in the composting of CM. Instead, Takahashi et al. (2016) reported that P mineralisation was influenced by factors

such as particle size and moisture content of compost, with increasing moisture levels leading to increased P bioavailability.

Contrastingly, there are significant variations in K values, with the maximum value in CM-WO (2.3%), followed by CM-M (1.9%), CM-P (1.8%), and CM-MP (0.08%). While CM-WO, CM-M, and CM-P show considerably high K content, CM-MP demonstrates exceptionally low K content. This does seem to depend on the combination of plant-derived materials and livestock manure as bulking agents, which induces a substantial increase in organic matter availability and creates gaps between compost particles. It is important to highlight the fact that K is not bonded to organic matter (Marschner, 1995), thus contributing to leaching between the particle gaps due to its high water solubility (Chang et al., 2022; Eghball et al., 2002; Santos et al., 2016). Additionally, Kutu and Masowa (2018) discovered that different initial composting feedstock reveals significantly different net K mineralisation rates. These reviews emphasise that the role of bulking agents extends beyond achieving an optimal C/N, also influencing the specific nutrient composition of compost with the objective of meeting plant requirements.

Bulk Density and Moisture Content

The structural characteristics of compost are determined by its bulk density, with the suggested target being to maintain BD below 400 kg/m³ for optimal results. However, BD data are not commonly recorded in the selected composting studies; hence, only CM-WO and CM-P generated sufficient data for comparison. The results show that an average BD of 521.8 kg/m³ and 401.3 kg/m³ for CM-WO and CM-P, respectively. The higher BD value observed in CM-WO is attributed to the absence of a bulking agent during composting. The consequences include reduced particle gaps and poor pile aeration, thus contributing to a slower decomposition process (Azim et al., 2018). Maintaining control over BD is crucial for creating a conducive environment for microbial decomposition and the transformation of organic matter into a nutrient-rich compost.

Optimal moisture content maintains a thin water film on particle surfaces, acting as the main site for microbial activity (Azim et al., 2018). The MC of all bulking agent categories falls within the ideal range of 40-60%, indicating that MC is not significantly affected by feedstock composition but rather by an external force, particularly the manual addition of water during the composting process (Azim et al., 2018). The control of MC during the composting process is pivotal to providing an adequate microbial site for optimal degradation.

Composting Duration

Composting often perceived as a time-consuming process, which drives preference for shorter maturation periods (Wong et al., 2017). The discrepancy in CD among different

bulking agent categories is shown in Table 2, with CM-M exhibiting the shortest duration (65.3 days), followed by CM-P (80.7 days), CM-MP (81 days), and CM-WO (98.7 days). The prolonged CD observed in CM-WO can be attributed to the negative effects of composting without a bulking agent, resulting in slower decomposition of organic matter and delayed maturation. This mainly happens due to an unfavourable C/N balance, which disrupts the sufficiency of primary nutrients required by composting microbes for a shorter maturation period (Haug, 2018 ; Zhou, 2014). In contrast, the short CD of CM-M could be well justified by the existing nutrient and microbial variations in the manure substrates, which benefit from the co-composting of multiple types of manure (Risberg et al., 2017; Sun et al., 2015). Additionally, the combination of CM with other manures may be favourable to microbial activity that produces heat (Hwang et al., 2020), which successfully accounts for the rapid degradation process and faster maturation reach (Bonanomi et al., 2016; Das et al., 2017). The choice of bulking agent significantly affects compost maturation time.

AEROBIC COMPOSTING METHODS OF CHICKEN MANURE COMPOSTING

There are different methods used for composting; the most adapted ones are in-vessel and windrow systems. This section will reveal the operation, advantages, and challenges, as well as the economic considerations of in-vessel and windrow systems in the aerobic composting of CM.

Windrow Composting Method

Windrow composting is the process of piling biodegradable organic matter into long, trapezoidal-shaped rows (Gould et al., 2013) of a certain length, height, and width depending on the available space and resources in that specific area (Andersen et al., 2010; Vergara & Silver, 2019). Windrow composting is classified into three types: turned windrow system (TWS), passively aerated windrow system (PAWS), and forced aerated static windrow (FASW) (Dincă et al., 2019). For TWS, a turning machine is typically employed to enhance oxygen flow and porosity, decrease moisture content, disperse hotter and colder areas of the pile, and boost microbial activity (Kong et al., 2018 ; Manyapu et al., 2017). This procedure does not require complicated technology and is cost-effective, but it demands labour, difficulty in maintaining moisture and temperature as well and substantial N loss was discovered (Manyapu et al., 2017; Ekinci et al., 2022). In PAWS, the pile must be a homogenous, low-density mixture (Michel et al., 2022a) with perforated pipes installed at the bottom of the windrow to ensure convective aeration throughout the pile (Lynch & Cherry, 1996; Patwa et al., 2020). The piles are not turned, and no forced aeration equipment is utilised, removing much of the capital and running costs associated with the previous method (Dincă et al., 2019). In contrast, FASW systems applied air

blowers connected to perforated pipes to force aeration, allowing airflow to be regulated through controlled frequency and duration of the blowers (Michel et al., 2022b). FASW is a well-established technology that allows higher and more uniform oxygen levels, removes excess heat and reduces greenhouse gas emissions (Michel et al., 2022b; Ekinci et al., 2022). In contrast, the common drawbacks of FASW are excessive drying of piles and difficulties in regulating moisture levels.

In-Vessel Composting Method

Composting technologies have undergone long-term improvements from static heaps and windrow composting to artificial intelligence-assisted in-vessel composting systems (Zhou et al., 2022). In-vessel composting can be applied regardless of the seasonal variation and geographical region, as the compost pile is fully controlled artificially within an enclosed system, such as in a building, container, or vessel. Different types of in-vessel systems have been built using fixed, agitated, or rotating bioreactors, which could accommodate either solid or liquid-state compost materials. This controlled composting system is getting exponentially increasing interest as mature composts could be produced more efficiently within a short time (Ajmal et al., 2020a). Besides, simultaneous handling of waste with minimal space could be employed with the production of high-quality mature compost containing fewer pathogenic concerns and lesser greenhouse gas emissions (Cekmecelioglu et al., 2005; Mu et al., 2017; Pandey et al., 2016). During composting, regulating temperature, moisture, and oxygen levels is vital in ensuring microbial degradation efficiency (Ajmal et al., 2020a; Oazana et al., 2018), which could be easily monitored via in-vessel systems. However, constant heating under temperature-controlled conditions may result in increased temperature for an extended length of time, reducing the nutritional content of compost (Ajmal et al., 2020b). Another downside of in-vessel composting is the high mechanical energy consumption that necessitates a large budget (Alkoaik et al., 2018; Mu et al., 2017).

Effects of Windrow and In-Vessel Composting Methods on Compost Physicochemical Parameters

Windrow and in-vessel composting methods have been widely applied across different continents, as depicted in Figure 2, which integrates data from 125 individual treatments spanning from the year 2014 to 2023 on the aerobic composting methods of CM. Fareast Asia stands out with the highest number of studies compared to the other continents, with 46 studies employing the in-vessel method and only 13 utilizing the windrow method. Similarly, the trend is observed across other continents, indicating a preference for the in-vessel approach over the windrow method, except for North America. In North America, two studies applied the windrow method, while only one employed the in-vessel method.

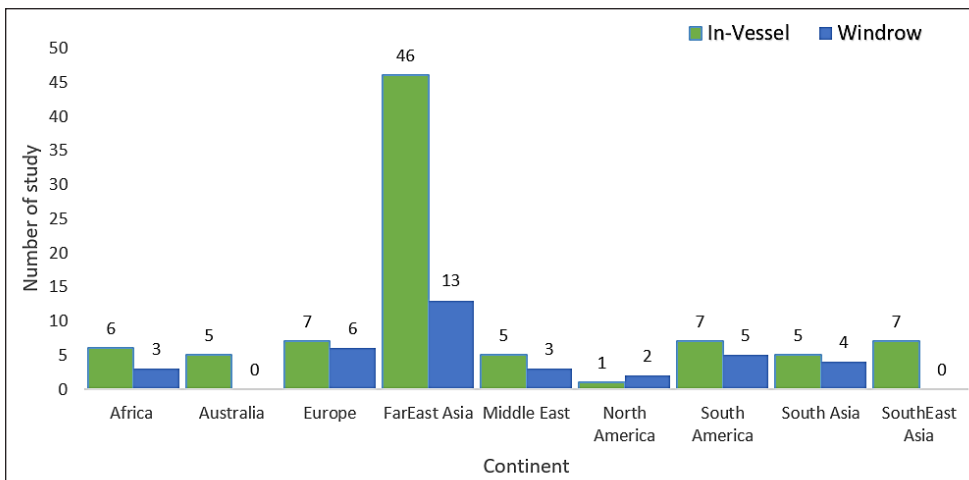


Figure 2. In-vessel and windrow composting systems of CM aerobic composting practised in different continents (n = 125)

The summary statistics for physicochemical parameters of final CM compost; comparison between in-vessel and windrow composting methods are presented in Table 3. Only two bulking agent categories, CM-WO and CM-P, which have more than three treatments ($n > 3$) each for both in-vessel and windrow systems, will be considered for comparison and further discussion. The in-vessel method applied for both CM-WO and CM-P exhibits a closer to ideal C/N than the windrow method. This discrepancy may be due to the capacity of the in-vessel method to control temperature within the compost pile, which is consistent with the findings of Ajmal et al. (2020b), who found that temperature is a significant factor influencing C/N of final compost. Aeration and moisture are other factors influencing the C/N of compost, which are feasible to control during in-vessel composting systems. Lack of aeration within the compost pile will result in anaerobic conditions, hindering the microbial degradation process, which induces negative net mineralisation (Gondek et al., 2020). Additionally, aeration may also be hindered when the moisture level exceeds 65%, as the thick moisture film surrounding the particles slows down oxygen transfer (Oshins et al., 2022). In contrast, the windrow method applied for CM-P shows a significantly higher average EC value (5.8 dS m^{-1}) compared to the ideal range, while the EC for the in-vessel method was superior (2.9 dS m^{-1}). This proved that the in-vessel method produced a better EC value in compost, which may result from the frequent pile turning and homogeneous distribution of salts. Furthermore, effective control of aeration, temperature, and moisture during the in-vessel process could also contribute to improved EC value of the final compost.

The in-vessel and windrow methods for CM-WO show inconsistent results for N content, whereas P and K contents show consistent patterns for both methods. The average P and K values

Table 3
Variable summary statistics for physicochemical parameters of final CM compost; comparison between in-vessel and windrow composting methods

Bulkling Agent Category	Composting Method	Summary statistics	Physicochemical Parameter									
			C/N	pH	EC (dS m ⁻¹)	N (%)	P (%)	K (%)	PS (cm)	BD (kg/m ³)	MC (%)	CD (day)
None (n = 14)	In-Vessel (CM-WO-IV)	Mean ± SD	13.9 ± 9.4 (n = 9)	7.4 ± 0.4 (n = 9)	2.6 ± 1.9 (n = 5)	2.5 ± 0.9 (n = 9)	1.2 ± 0.6 (n = 6)	1.2 ± 0.7 (n = 5)	0.5 (n = 1)	450 ± 282.8 (n = 2)	41.5 ± 21.6 (n = 8)	110.1 ± 106.7 (n = 9)
		Min	6.3	6.4	0.3	1.0	0.6	0.5	0.5	250	15	9
		Max	37.2	8.0	5.4	4.0	2.2	1.9		650	70	365
		Mean ± SD	13.5 ± 4.7 (n = 5)	8.0 ± 0.9 (n = 5)	14.7 ± 20.4 (n = 2)	2.2 ± 1.8 (n = 5)	1.4 ± 1.0 (n = 4)	4.4 ± 1.3 (n = 3)		675 ± 191 (n = 2)	88.4 (n = 1)	107.3 ± 66.5 (n = 4)
Plant-derived (n = 73)	In-Vessel (CM-P-IV)	Min	7.6	6.6	0.2	0.3	0.00004	3.5		539.9	88.4	56
		Max	18.1	8.9	29.1	4.3	2.3	5.9		810		205
		Mean ± SD	18.6 ± 7.9 (n = 51)	8.2 ± 0.9 (n = 52)	2.9 ± 1.9 (n = 27)	1.6 ± 1.0 (n = 29)	1.6 ± 2.4 (n = 21)	1.6 ± 1.9 (n = 19)	0.9 ± 0.6 (n = 4)	396.8 ± 114.8 (n = 6)	50.7 ± 14.1 (n = 27)	66.5 ± 39.1 (n = 53)
		Min	2.2	6.4	0.003	0.2	0.01	0.001	0.03	260	18.8	0.9
	Windrow (CM-P-W)	Max	43.1	11.0	8.5	5.0	11.3	8.5	1.5	500	67	168
		Mean ± SD	15.1 ± 5.3 (n = 19)	8.0 ± 0.9 (n = 17)	5.8 ± 3.7 (n = 13)	2.1 ± 1.0 (n = 17)	1.7 ± 1.7 (n = 13)	2.4 ± 1.9 (n = 13)	0.6 (n = 1)	415 ± 219.2 (n = 2)	43.2 ± 16.5 (n = 13)	123.2 ± 139.8 (n = 19)
		Min	6.9	6.0	0.1	0.1	0.0002	0.02	0.6	260	17.5	20
		Max	24.8	9.1	11.7	4.0	5.6	4.6		570	67.6	600

Note. CM-WO-IV, chicken manure composted without bulking agent using in-vessel method; CM-WO-W, chicken manure composted without bulking agent using windrow method; CM-P-IV, chicken manure co-composted with plant-derived materials using in-vessel method; CM-P-W, chicken manure co-composted with plant-derived materials using windrow method; SD, standard deviation; C/N, carbon-to-nitrogen ratio; EC, electrical conductivity; N, nitrogen; P, phosphorus; K, potassium; PS, particle size; BD, bulk density; MC, moisture content; CD, composting duration. Number of data reviewed for this analysis, n = 87

from the in-vessel method applied to CM-WO and CM-P are both lower than those from the windrow method, with K values showing a significantly higher difference between the two methods. Specifically, the K value for CM-WO-W is higher by 3.2% than CM-WO-IV, while CM-P-W is higher by 0.8% than CM-P-IV. Iyengar and Bhawe (2006) discovered that in-vessel composting produced composts with low K levels; thus, they proposed that an external source of K be added to increase the quality of compost. The reduced nutrient levels could be attributed to the downside of K, which could be easily drained as leachate. Similarly, Hu et al. (2009) found that the reduced concentrations of P and K at the end of in-vessel composting do appear to be associated with leaching, as these elements are non-volatile. Therefore, it is advisable to reintroduce leachate from in-vessel composting back into the compost pile (Iyengar & Bhawe, 2006). The observed nutrient variations emphasise the importance of considering the composting method to achieve compost with adequate plant-available nutrients.

The in-vessel composting methods for both CM-WO and CM-P exhibit superior BD values when compared with the windrow method. The variation in BD arises from factors such as moisture content and particle size. The in-vessel system excels as it provides consistent regulation in pile moisture levels, and particle size screening is often conducted before the composting process begins. Larger particles tend to degrade more slowly due to their lower surface area available for microbial activity (Truong & Marschner, 2018). In contrast, the windrow composting method is typically conducted in large batches without particle size screening and pile moisture control. This may cause uneven compaction over time, making turning operations more challenging and less effective.

The average CD of CM-WO, either using the in-vessel or windrow method, does not show a significant difference (110.1 and 107.3 days, respectively), while the average CD for CM-P shows a remarkable difference with CM-P-IV (66.5 days) and CM-P-W (123.2 days). In-vessel composting is more active in organic matter breakdown and reaches maturity faster than windrow composting. The duration of the composting process is determined by the inherent nature of the decomposed organic matter, and the process efficiency is determined by the amount of aeration and agitation (Ajmal et al., 2020a). Accordingly, the aeration homogeneity provided by frequent turning equipped with in-vessel composting technology contributed to a shorter CD. In addition, moisture is also necessary to support the metabolic processes of the microbes. Both methods exhibited optimal MC; however, it is worth noting that higher MC was recorded in CM-P-IV (49.1%) than in CM-P-W (43.2%)

Active composting will change depending on the amount of natural moisture or water added to the compost. Since moisture control is more easily adaptable with the in-vessel method compared to the windrow method, this approach demonstrates improved moisture levels and shorter composting duration. In summary, aeration rate, temperature, and moisture content are the important parameters that affect the composting duration and ultimately affect the quality of the compost product.

CORRELATION BETWEEN THE PHYSICOCHEMICAL PARAMETERS OF COMPOST

The interactions of physicochemical parameter dynamics were proven to have significant effects on one another in numerous prior studies (Ho et al., 2022; Ogunwande et al., 2008; Shi et al., 2020). For that reason, the correlation between the physicochemical parameters will be further discussed by reviewing data from Tables 2 and 3. From Table 2, CM-WO has the lowest average C/N (12.9) and the highest average K (2.3 %), and vice versa for CM-MP. Table 3 also shows the same pattern, where both higher C/N in CM-WO and CM-P retain lower K content in the compost pile. Therefore, it was observed that C/N exhibited a notable antagonistic correlation with K content. This review aligns with studies conducted by Zhou (2017) and Khalid et al. (2014). Zhou (2017) found that a compost pile with low C/N has higher K concentrations throughout the composting process of pig manure and edible fungus residue. This could be attributed to the increased mineralisation rate at low C/N, consequently increasing the amount of K in the compost pile. The increase in K mineralisation might also be related to acid production by microbes at low C/N (Garg et al., 2006 ; Dume et al., 2023). Ogunwande et al. (2008) have also found that C/N has a significant effect on K content during the composting process. The porosity of the compost pile could also be indicative of this negative correlation. Lack of bulking agent in CM-WO has resulted in low porosity, thus less K leaching as it is highly soluble in water (Chang et al., 2022; Santos et al., 2016).

The increase in N mineralisation was observed to cause a significant decline in pH value, as shown in Table 2, where N concentration for CM-P (the highest pH) is significantly lower than the other bulking agent categories. Azim et al. (2018) reviewed that the same outcome was found in several studies, which could be attributable to the high amount of hydrogen ions released due to increased nitrification, creating an acidic environment (Fang & Wong, 1999; McKinley & Vestal, 1985). Shi et al. (2020) stated that this antagonistic correlation indicates an imbalance in the transfer of protons and electrons during the biochemical process of composting. Furthermore, the elevated pH value suggests a significant presence of ammonia, which also hinders the nitrification process and leads to increased N losses (Chen et al., 2011 ; Wang et al., 2015). Cáceres et al. (2018) mentioned that nitrification can be inhibited due to extreme pH values (below 5 or above 8); similarly, this study also showed that pH values that stay below 8.0 corresponded to higher N values at the end of composting.

The present study confirmed previous results on how compost particle size is associated with bulk density values (Głąb et al., 2020; Ho et al., 2022). Khater (2015) studied the porosity and bulk density of cattle manure and sugar cane residues and found that sugar cane has higher porosity and lower bulk density than cattle manure. This affirms that materials with high porosity generally display low bulk density values, consequently influencing compost pile density. Table 2 illustrates that CM-P has a higher average

particle size, resulting in a lower and better average BD value than CM-WO. Therefore, it can be concluded that the high porosity of plant-derived materials in CM-P contributed to better BD values compared to CM-WO, which lacks the incorporation of a bulking agent. Shorter CD is associated with higher MC, as illustrated in Table 2, where the highest MC in CM-WO had the longest CD, and vice versa for the lowest MC in CM-M. Similarly, Nada (2015) reported that MC has an insignificant effect on the physicochemical quality parameters of compost. Changes in MC are closely related to aeration and temperature levels within the compost pile. Past studies have indicated that approximately 90 % of heat dissipation can be attributed to water evaporation in an aerated static pile system (Sasaay et al., 1997). The lack of bulking agent incorporation in CM-WO has resulted in inadequate space between the compost particles, thus preventing proper aeration within the composting pile. This influences the presence of oxygen for aerobic microbial activity and explains the long average CD of 98.7 days in CM-WO. Adequate pore spaces are crucial as they allow for proper aeration and prevent compaction within the compost pile for a more efficient composting process.

CONCLUSION

The comprehensive review provides insights into the recent developments in the aerobic composting of CM, particularly focusing on the effects of different bulking agents and composting methods on the physicochemical parameters. Plant-derived materials and livestock manure emerged as the most commonly utilised bulking agents in CM composting, playing crucial roles for optimising C/N, EC, K, BD and CD. The windrow method provides better retention of P and K, while the in-vessel method performed well in improving C/N ratio, EC, BD, MC and CD. Negative correlations were observed between C/N ratio and N, K and pH values, respectively, as well as between BD and particle size. A positive correlation was observed between CD and MC. These reviews highlight the importance of selecting suitable bulking agents and composting methods, as their interactions directly influence CM compost quality.

LIMITATIONS

This comprehensive review provides valuable insights into the effects of different bulking agents and composting methods. However, several limitations should be acknowledged. First, the review did not account for variations in composting duration, turning frequency, or ambient climatic conditions across the selected studies. Such heterogeneity may constrain the direct comparability of results among different experimental setups. Second, the analysis only focused on physicochemical parameters, without consideration of microbiological dynamics (e.g., pathogen reduction, microbial community succession) or gaseous emissions (e.g., ammonia, greenhouse gases). These factors are essential for a holistic evaluation of

composting performance and environmental impact. Third, the majority of the included studies were conducted under controlled or pilot-scale conditions, which may not fully represent the operational challenges and feedstock variability inherent in full-scale, real-world composting facilities.

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REFERENCES

- Agyarko-Mintah, E., Cowie, A., Singh, B. P., Joseph, S., Van Zwieten, L., Cowie, A., Smillie, R. (2017). Biochar increases nitrogen retention and lowers greenhouse gas emissions when added to composting poultry litter. *Waste Management*, 61, 138-149. <https://doi.org/10.1016/j.wasman.2016.11.027>
- Ajmal, M., Aiping, S., Uddin, S., Awais, M., Faheem, M., Ye, L., Shi, Y. (2020a). A review on mathematical modelling of in-vessel composting process and energy balance. *Biomass Conversion and Biorefinery*, 12, 1-13. <https://doi.org/10.1007/s13399-020-00883-y>
- Ajmal, M., Aiping, S., Awais, M., Ullah, M. S., Saeed, R., Uddin, S., Zihao, X. (2020b). Optimisation of pilot-scale in-vessel composting process for various agricultural wastes on elevated temperature by using Taguchi technique and compost quality assessment. *Process Safety and Environmental Protection*, 140, 34-45. <https://doi.org/10.1016/j.psep.2020.05.001>
- Alkoaik, F. N., Abdel-Ghany, A. M., Rashwan, M. A., Fulleros, R. B., Ibrahim, M. N. (2018). Energy analysis of a rotary drum bioreactor for composting tomato plant residues. *Energies*, 11(2), Article 449. <https://doi.org/10.3390/en11020449>
- Andersen, J. K., Boldrin, A., Samuelsson, J., Christensen, T. H., Scheutz, C. (2010). Quantification of Greenhouse Gas Emissions from Windrow Composting of Garden Waste. *Journal of Environmental Quality*, 39(2). <https://doi.org/10.2134/jeq2009.0329>
- Awasthi, M. K., Pandey, A. K., Bundela, P. S., Wong, J. W., Li, R., Zhang, Z. (2016). Co-composting of gelatin industry sludge combined with organic fraction of municipal solid waste and poultry waste employing zeolite mixed with enriched nitrifying bacterial consortium. *Bioresource Technology*, 213, 181-189. <https://doi.org/10.1016/j.biortech.2016.02.026>
- Azim, K., Soudi, B., Boukhari, S., Perissol, C., Roussos, S., Thami Alami, I. (2018). Composting parameters and compost quality: a literature review. *Organic Agriculture*, 8(2), 141-158. <https://doi.org/10.1007/s40093-017-0157-7>
- Biala, J., Lovrick, N., Rowlings, D., & Grace, P. (2016). Greenhouse-gas emissions from stockpiled and composted dairy-manure residues and consideration of associated emission factors. *Animal Production Science*, 56(9), 1432-1441. <https://doi.org/10.1071/AN16009>

- Bonanomi, G., Filippis, F. D., Cesarano, G., Storia, A. L., Ercolini, D., Scala, F. (2016). Organic farming induces changes in soil microbiota that affect agroecosystem functions. *Soil Biol. Biochem*, 103, 327-336. <https://doi.org/10.1016/j.soilbio.2016.09.005>
- Butterly, C. R., Baldock, J. A., Tang, C. (2013). The contribution of crop residues to changes in soil pH under field conditions. *Plant and Soil*, 366(1-2). <https://doi.org/10.1007/s11104-012-1422-1>
- Cáceres, R., Malińska, K., Marfà, O. (2018). Nitrification within composting: A review. *Waste Management*, 72, 119-137. <https://doi.org/10.1016/j.wasman.2017.10.049>
- Calisti, R., Regni, L., Proietti, P. (2020). Compost-recipe: A new calculation model and a novel software tool to make the composting mixture. *Journal of Cleaner Production*, 270. <https://doi.org/10.1016/j.jclepro.2020.122427>
- Carmo, D. L. D., Lima, L. B. D., Silva, C. A. (2016). Soil fertility and electrical conductivity affected by organic waste rates and nutrient inputs. *Revista Brasileira de Ciência do Solo*, 40, Article e0150152. <https://doi.org/10.1590/18069657rbcs20150152>
- Cekmecelioglu, D., Demirci, A., Graves, R. E., Davitt, N. H. (2005). Applicability of optimised in-vessel food waste composting for windrow systems. *Biosystems engineering*, 91(4), 479-486. <https://doi.org/10.1016/j.biosystemseng.2005.04.013>
- Chai, E. W., H'ng, P. S., Peng, S. H., Wan-Azha, W. M., Chin, K. L., Chow, M. J., Wong, W. Z. (2013). Compost feedstock characteristics and ratio modelling for organic waste materials co-composting in Malaysia. *Environmental Technology*, 34(20), 2859-2866. <https://doi.org/10.1080/09593330.2013.795988>
- Chang, C. T., Negi, S., Rani, A., Hu, A. H., Pan, S. Y., Kumar, S. (2022). Food waste and soybean curd residue composting by black soldier fly. *Environmental Research*, 214, Article 113792. <https://doi.org/10.1016/j.envres.2022.113792>
- Chen, L., de Haro Marti, M., Moore, A., & Falen, C. (2011). *Dairy compost production and use in Idaho*. University of Idaho Extension.
- Choi, K. (1999). Optimal operating parameters in the composting of swine manure with wastepaper. *Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes*, 34(6). <https://doi.org/10.1080/03601239909373240>
- Chowdhury, M. A., de Neergaard, A., Jensen, L. S. (2014). Potential of aeration flow rate and bio-char addition to reduce greenhouse gas and ammonia emissions during manure composting. *Chemosphere*, 97, 16-25. <https://doi.org/10.1016/j.chemosphere.2013.10.030>
- Congilosi, J. L., Aga, D. S. (2021). Review on the fate of antimicrobials, antimicrobial resistance genes, and other micropollutants in manure during enhanced anaerobic digestion and composting. *Journal of Hazardous Materials*, 405, Article 123634. <https://doi.org/10.1016/j.jhazmat.2020.123634>
- Das, S., Jeong, S. T., Das, S., Kim, P. J. (2017). Composted cattle manure increases microbial activity and soil fertility more than composted swine manure in a submerged rice paddy. *Frontiers in Microbiology*, 8, Article 1702. <https://doi.org/10.3389/fmicb.2017.01702>
- Dincă, M. N., Ferdeş, M., Paraschiv, G., Ungureanu, N., Zăbavă, B., Ionescu, M., Moiceanu, G. (2019). Recovery of organic waste through composting process. *Acta Technica Corviniensis-Bulletin of Engineering*, 12(1).

- Dume, B., Hanc, A., Svehla, P., Michal, P., Chane, A. D., Nigussie, A. (2023). Composting and vermicomposting of sewage sludge at various C/Ns: technological feasibility and end-product quality. *Ecotoxicology and Environmental Safety*, 263, Article 115255. <https://doi.org/10.1016/j.ecoenv.2023.115255>
- Eckhardt, D. P., Redin, M., Santana, N. A., Conti, L. D., Dominguez, J., Jacques, R. J. S., Antonioli, Z. I. (2018). Cattle manure bioconversion effect on the availability of nitrogen, phosphorus, and potassium in soil. *Revista Brasileira de Ciência do Solo*, 42, Article e0170327. <https://doi.org/10.1590/18069657rbcs20170327>
- Eghball, B., Wienhold, B. J., Gilley, J. E., Eigenberg, R. A. (2002). Mineralisation of manure nutrients. *Journal of Soil and Water Conservation*, 57(6). <https://doi.org/10.1080/00224561.2002.12457480>
- Ekinci, K., Tosun, İ., Sülük, K., Şevik, F., Kumbul, B. S., & Bitrak, N. B. (2022). Evaluation of forced aerated and turned pile composting of rose oil processing wastes. *Environment Protection Engineering*, 48(1). <https://doi.org/10.37190/epe220107>
- Estévez-Schwarz, I., Seoane-Labandeira, S., Núñez-Delgado, A., López-Mosquera, M. E. (2012). Production and characterisation of compost made from garden and other waste. *Polish Journal of Environmental Studies*, 21(4). <https://acta.fih.upt.ro/pdf/2019-1/ACTA-2019-1-24.pdf>
- Fang, M., Wong, J. W. C. (1999). Effects of lime amendment on availability of heavy metals and maturation in sewage sludge composting. *Environmental Pollution*, 106(1), 83-89. [https://doi.org/10.1016/S0269-7491\(99\)00056-1](https://doi.org/10.1016/S0269-7491(99)00056-1)
- Fernando, R. L. S. (2019). Solid waste management of local governments in the Western Province of Sri Lanka: An implementation analysis. *Waste Management*, 84, 194-203. <https://doi.org/10.1016/j.wasman.2018.11.030>
- Food and Agriculture Organisation. (2023). *Statistical yearbook: World food and agriculture*. FAO. <https://www.fao.org/3/cc8166en/cc8166en.pdf>
- Garg, P., Gupta, A., Satya, S. (2006). Vermicomposting of different types of waste using *Eisenia foetida*: A comparative study. *Bioresource Technology*, 97(3). <https://doi.org/10.1016/j.biortech.2005.03.009>
- Głąb, T., Żabiński, A., Sadowska, U., Gondek, K., Kopeć, M., Mierzwa-Hersztek, M., Stanek-Tarkowska, J. (2020). Fertilisation effects of compost produced from maize, sewage sludge and biochar on soil water retention and chemical properties. *Soil and Tillage Research*, 197, Article 104493. <https://doi.org/10.1016/j.still.2019.104493>
- Gondek, M., Weindorf, D. C., Thiel, C., Kleinheinz, G. (2020). Soluble salts in compost and their effects on soil and plants: A review. *Compost Science & Utilisation*, 28(2), 59-75. <https://doi.org/10.1080/1065657X.2020.1772906>
- Gould, J. C., Rossano, M. G., Lawrence, L. M., Burk, S. v., Ennis, R. B., Lyons, E. T. (2013). The effects of windrow composting on the viability of *Parascaris equorum* eggs. *Veterinary Parasitology*, 191(1-2). <https://doi.org/10.1016/j.vetpar.2012.08.017>
- Grisey, E., Aleya, L. (2015). Prolonged aerobic degradation of shredded and pre-composted municipal solid waste: report from a 21-year study of leachate quality characteristics. *Environmental Science and Pollution Research*, 23(1), 800-815. <https://doi.org/10.1007/s11356-015-5315-8>

- Guo, H. nan, Liu, H. tao, Wu, S. (2022). Immobilisation pathways of heavy metals in composting: Interactions of microbial community and functional gene under varying C/N ratios and bulking agents. *Journal of Hazardous Materials*, 426, Article 128103. <https://doi.org/10.1016/j.jhazmat.2021.128103>
- Heyman, H., Bassuk, N., Bonhotal, J., Walter, T. (2019). Compost quality recommendations for remediating urban soils. *International Journal of Environmental Research and Public Health*, 16(17). <https://doi.org/10.3390/ijerph16173191>
- Ho, T. T. K., Le, T. H., Tran, C. S., Nguyen, P. T., Thai, V. N., Bui, X. T. (2022). Compost to improve sustainable soil cultivation and crop productivity. *Case Studies in Chemical and Environmental Engineering*, 6, Article 100211. <https://doi.org/10.1016/j.cscee.2022.100211>
- Haug, R. T. (2018). *The practical handbook of compost engineering*. Routledge. <https://doi.org/10.1201/9780203736234>
- Hu, Z., Lane, R., Wen, Z. (2009). Composting clam processing wastes in a laboratory-and pilot-scale in-vessel system. *Waste Management*, 29(1), 180-185. <https://doi.org/10.1016/j.wasman.2008.02.016>
- Huang, J., Yu, Z., Gao, H., Yan, X., Chang, J., Wang, C., Zhang, L. (2017). Chemical structures and characteristics of animal manures and composts during composting and assessment of maturity indices. *PLOS ONE*, 12(6), Article e0178110. <https://doi.org/10.1371/journal.pone.0178110>
- Hubbe, M. A., Nazhad, M., Sánchez, C. (2010). Composting as a way to convert cellulosic biomass and organic waste into high-value soil amendments: A review. *BioResources*, 5(4), 2808-2854. <https://doi.org/10.15376/biores.5.4.2808-2854>
- Hwang, H. Y., Kim, S. H., Kim, M. S., Park, S. J., & Lee, C. H. (2020). Co-composting of chicken manure with organic wastes: characterisation of gases emissions and compost quality. *Applied Biological Chemistry*, 63, 1-10. <https://doi.org/10.1186/s13765-019-0483-8>
- Iyengar, S. R., Bhave, P. P. (2006). In-vessel composting of household wastes. *Waste Management*, 26(10), 1070-1080. <https://doi.org/10.1016/j.wasman.2005.06.011>
- Kaza, S., Yao, L., Bhada-Tata, P., Van Woerden, F. (2018). *What a waste 2.0: A global snapshot of solid waste management to 2050*. World Bank Publications. <https://doi.org/10.1596/978-1-4648-1329-0>
- Khalid, I., Nadeem, A., Ahmed, R., Husnain, A. (2014). Conjunctive and mineralisation impact of municipal solid waste compost and inorganic fertiliser on lysimeter and pot studies. *Environmental Technology*, 35(4), 487-498. <https://doi.org/10.1080/09593330.2013.833641>
- Khan, N., Clark, I., Sánchez-Monedero, M. A., Shea, S., Meier, S., Bolan, N. (2014). Maturity indices in co-composting of chicken manure and sawdust with biochar. *Bioresource Technology*, 168, 245-251. <https://doi.org/10.1016/j.biortech.2014.02.123>
- Khater, E. S. G. (2015). Some physical and chemical properties of compost. *International Journal of Waste Resources*, 5(1), 72-79. <https://doi.org/10.4172/2252-5211.1000172>
- Kim, J. D., Park, J. S., In, B. H., Kim, D., Namkoong, W. (2008). Evaluation of pilot-scale in-vessel composting for food waste treatment. *Journal of Hazardous Materials*, 154(1-3), 272-277. <https://doi.org/10.1016/j.jhazmat.2007.10.023>

- Kong, Z., Wang, X., Liu, Q., Li, T., Chen, X., Chai, L., Liu, D., Shen, Q. (2018). Evolution of various fractions during the windrow composting of chicken manure with rice chaff. *Journal of Environmental Management*, 207. <https://doi.org/10.1016/j.jenvman.2017.11.023>
- Kutu, F. R., Masowa, M. M. (2018). Nitrogen and potassium mineralisation from winery solid waste composts in sandy and sandy loam soils. *Archives of Agronomy and Soil Science*, 64(8), 1094-1105. <https://doi.org/10.1080/03650340.2017.1414195>
- Lauber, C. L., Hamady, M., Knight, R., Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, 75(15), 5111-5120. <https://doi.org/10.1128/AEM.00335-09>
- Li, S., Li, D., Li, J., Li, Y., Li, G., Zang, B., & Li, Y. (2018). Effect of spent mushroom substrate as a bulking agent on gaseous emissions and compost quality during pig manure composting. *Environmental Science and Pollution Research*, 25(13), 12398-12406. <https://doi.org/10.1007/s11356-018-1450-3>
- Liu, Y., Wang, W., Xu, J., Xue, H., Stanford, K., McAllister, T. A., Xu, W. (2018). Evaluation of compost, vegetable and food waste as amendments to improve the composting of NaOH/NaClO-contaminated poultry manure. *PLOS ONE*, 13(10). <https://doi.org/10.1371/journal.pone.0205112>
- Lynch, N. J., Cherry, R. S. (1996). Design of passively aerated compost piles: Vertical air velocities between the pipes. *Biotechnology Progress*, 12(5). <https://doi.org/10.1021/bp960048>
- Manish, B., Richa, G., & Archana, T. (2013). Implementation of bulking agents in composting: A review. *Journal of Bioremediation & Biodegradation*, 4(7), Article 205. <https://doi.org/10.4172/2155-6199.1000205>
- Manyapu, V., Shukla, S., Kumar, S., Rajendra, K. (2017). In-vessel composting: A rapid technology for conversion of biowaste into compost. *Open Access International Journal of Sciences & Engineering*, 2(9). <https://doi.org/10.65521/oaijse.v2i9.2366>
- McKinley, V. L., & Vestal, J. R. (1985). Effects of different temperature regimes on microbial activity and biomass in composting municipal sewage sludge. *Canadian Journal of Microbiology*, 31(10), 919-925. <https://doi.org/10.1139/m85-172>
- Mehta, C. M., Khunjar, W. O., Nguyen, V., Tait, S., & Batstone, D. J. (2015). Technologies to recover nutrients from waste streams: A critical review. *Critical Reviews in Environmental Science and Technology*, 45(4), 385-427. <https://doi.org/10.1080/10643389.2013.866621>
- Michel, F., O'Neill, T., Rynk, R., Gilbert, J., Wisbaum, S., & Halbach, T. (2022). Passively aerated composting methods, including turned windrows. In R. Rynk (Ed.), *The composting handbook* (pp. 159-196). Academic Press. <https://doi.org/10.1016/B978-0-323-85602-7.00007-8>
- Michel, F., O'Neill, T., Rynk, R., Gilbert, J., Smith, M., Aber, J., Keener, H. (2022b). Forced aeration composting, aerated static pile, and similar methods. In R. Rynk (Ed.), *The composting handbook* (pp. 197-269). Academic Press. <https://doi.org/10.1016/B978-0-323-85602-7.00007-8>
- Mu, D., Horowitz, N., Casey, M., Jones, K. (2017). Environmental and economic analysis of an in-vessel food waste composting system at Kean University in the US. *Waste Management*, 59, 476-486. <https://doi.org/10.1016/j.wasman.2016.10.026>

- Nada, W. M. (2015). Stability and maturity of maize stalks compost as affected by aeration rate, C/N and moisture content. *Journal of Soil Science and Plant Nutrition*, 15(3), 751-764. <https://doi.org/10.4067/S0718-95162015005000051>
- Nahm, K. H. (2003). Evaluation of the nitrogen content in poultry manure. *World's Poultry Science Journal*, 59(1), 77-88. <https://doi.org/10.1079/WPS20030004>
- Neher, D. A., Limoges, M. A., Weicht, T. R., Sharma, M., Millner, P. D., & Donnelly, C. (2020). Bacterial community dynamics distinguish poultry compost from dairy compost and non-amended soils planted with spinach. *Microorganisms*, 8(10), Article 1601. <https://doi.org/10.3390/microorganisms8101601>
- Nurin, A. Y., Tee, T. P., Chin, N. L., Nayan, N. (2025). Sustainable optimisation of co-composting horse and chicken manures using Response Surface Methodology (RSM). *Compost Science & Utilisation*, 32(1-2), 57-74. <https://doi.org/10.1080/1065657X.2025.2476397>
- Nurin, A. Y., Tee, T. P., Chin, N. L., Zainudin, M. H. M., Nayan, N. (2024). Optimisation of watermelon waste as a bulking agent for sustainable co-composting of livestock manures using response surface methodology. *Frontiers in Sustainable Food Systems*, 8, Article 1368970. <https://doi.org/10.3389/fsufs.2024.1368970>.
- Nyi, T., Varughese, P., bin Hj Bujang, M. I., Ra, K., Irianta, B., Sengxua, P., Sipaseuth, N., Harirah, A. A., bin Jantan, B., Salguero, S. M., Meunchang, P., Manh Quyet, V., Quang Hai, N., Moody, P., Jäkel, T. E., & Soda, W. (2017). *ASEAN guidelines on soil and nutrient management*. ASEAN Sectoral Working Group on Crops.
- Oagile, D., & Namasiku, M. (2010). Chicken manure-enhanced soil fertility and productivity: Effects of application rates. *Journal of Soil Science and Environmental Management*, 1(3), 46-54.
- Oazana, S., Naor, M., Grinshpun, J., Halachmi, I., Raviv, M., Saadi, I., Laor, Y. (2018). A flexible control system designed for lab-scale simulations and optimisation of composting processes. *Waste Management*, 72, 150-160. <https://doi.org/10.1016/j.wasman.2017.11.029>
- Ogunwande, G. A., Osunade, J. A., Adekalu, K. O., & Ogunjimi, L. A. O. (2008). Nitrogen loss in chicken litter compost as affected by carbon to nitrogen ratio and turning frequency. *Bioresource Technology*, 99(16), 7495-7503. <https://doi.org/10.1016/j.biortech.2008.02.020>
- Ogunwande, G. A. (2011). Carbon loss in chicken litter compost as affected by carbon to nitrogen ratio and turning frequency. *Ife Journal of Technology*, 20(1), 34-38. <https://ijt.oauife.edu.ng/index.php/ijt/article/view/77>
- Oshins, C., Michel, F., Louis, P., Richard, T. L., & Rynk, R. (2022). The composting process. In R. Rynk (Ed.), *The composting handbook* (pp. 51-101). Academic Press. <https://doi.org/10.1016/B978-0-323-85602-7.00008-X>
- Pace, M. G., Miller, B. E., & Farrell-Poe, K. L. (1995). *The composting process* (AG-WM-01). Utah State University Extension. https://digitalcommons.usu.edu/extension_histall/48/
- Pandey, P. K., Vaddella, V., Cao, W., Biswas, S., Chiu, C., Hunter, S. (2016). In vessel composting system for converting food and green wastes into pathogen free soil amendment for sustainable agriculture. *Journal of Cleaner Production*, 139, 407-415. <https://doi.org/10.1016/j.jclepro.2016.08.034>

- Paredes, C., Bernal, M. P., Cegarra, J., Roig, A., & Navarro, A. F. (1996). Nitrogen transformation during the composting of different organic wastes. In Van Cleemput, O., Hofman, G., Vermoesen, A. (Eds.), *Progress in nitrogen cycling studies* (pp. 121-125). Springer. https://doi.org/10.1007/978-94-011-5450-5_19
- Patwa, A., Parde, D., Dohare, D., Vijay, R., & Kumar, R. (2020). Solid waste characterisation and treatment technologies in rural areas: An Indian and international review. *Environmental Technology & Innovation*, 20, Article 101066. <https://doi.org/10.1016/j.eti.2020.101066>
- Rehman, K. u., Cai, M., Xiao, X., Zheng, L., Wang, H., Soomro, A. A., Zhou, Y., Li, W., Yu, Z., & Zhang, J. (2017). Cellulose decomposition and larval biomass production from the co-digestion of dairy manure and chicken manure by mini-livestock (*Hermetia illucens* L.). *Journal of Environmental Management*, 196, 458-465. <https://doi.org/10.1016/j.jenvman.2017.03.047>
- Risberg, K., Cederlund, H., Pell, M., Arthurson, V., & Schnürer, A. (2017). Comparative characterisation of digestate versus pig slurry and cow manure-chemical composition and effects on soil microbial activity. *Waste Management*, 61, 529-538. <https://doi.org/10.1016/j.wasman.2016.12.016>
- Rynk, R., van de Kamp, M., Willson, G. B., Singley, M. E., Richard, T. L., Kolega, J. J., Gouin, F. R., Laliberty, L., Kay, D., Murphy, D.W., Hoitink, H. A. J., & Brinton, W. F. (1992). *On-farm composting handbook* (NRAES-54). Northeast Regional Agricultural Engineering Service. <https://hdl.handle.net/1813/67142>
- Rynk, R., Schwarz, M., Richard, T. L., Cotton, M., Halbach, T., & Siebert, S. (2022). Compost feedstocks. In R. Rynk (Ed.), *The composting handbook* (pp. 103-157). Academic Press. <https://doi.org/10.1016/B978-0-323-85602-7.00005-4>
- Sánchez, Ó. J., Ospina, D. A., & Montoya, S. (2017). Compost supplementation with nutrients and microorganisms in composting process. *Waste Management*, 69, 136-153. <https://doi.org/10.1016/j.wasman.2017.08.012>
- Santos, A., Bustamante, M. A., Tortosa, G., Moral, R., & Bernal, M. P. (2016). Gaseous emissions and process development during composting of pig slurry: The influence of the proportion of cotton gin waste. *Journal of Cleaner Production*, 112, 81-90. <https://doi.org/10.1016/j.jclepro.2015.08.084>
- Sesay, A. A., Lasaridi, K., Stentiford, E., & Budd, T. (1997). Controlled composting of paper pulp sludge using the aerated static pile method. *Compost Science & Utilization*, 5(1), 82-96. <https://doi.org/10.1080/1065657X.1997.10701866>
- Shahbandeh, M. (2023). *Global chicken meat production 2022 & 2023, by selected country*. Statista. <https://www.statista.com/statistics/237597/leading-10-countries-worldwide-in-poultry-meat-production-in-2007/>
- Sharma, M., & Reynnells, R. (2016). Importance of soil amendments: Survival of bacterial pathogens in manure and compost used as organic fertilisers. *Microbiology Spectrum*, 4(4), Article PFS-0010-2015. <https://doi.org/10.1128/microbiolspec.PFS-0010-2015>
- Shi, M., Zhao, Y., Zhu, L., Song, X., Tang, Y., Qi, H., Cao, H., & Wei, Z. (2020). Denitrification during composting: Biochemistry, implication and perspective. *International Biodeterioration & Biodegradation*, 153, Article 105043. <https://doi.org/10.1016/j.ibiod.2020.105043>
- Silva, M. E. F., de Lemos, L. T., Nunes, O. C., & Cunha-Queda, A. C. (2014). Influence of the composition of the initial mixtures on the chemical composition, physicochemical parameters and humic-like substances content of composts. *Waste Management*, 34(1), 21-27. <https://doi.org/10.1016/j.wasman.2013.09.011>

- Singh, J., & Kalamdhad, A. S. (2015). Assessment of compost quality in agitated pile composting of water hyacinth collected from different sources. *International Journal of Recycling of Organic Waste in Agriculture*, 4(3), 175-183. <https://doi.org/10.1007/s40093-015-0097-z>
- Smith, J. L., & Doran, J. W. (2015). Measurement and use of pH and electrical conductivity for soil quality analysis. In *Methods for assessing soil quality* (pp. 161-169). Soil Science Society of America. <https://doi.org/10.2136/sssaspecpub49.c10>
- Song, C., Li, M., Jia, X., Wei, Z., Zhao, Y., Xi, B., Zhu, C., & Liu, D. (2014). Comparison of bacterial community structure and dynamics during the thermophilic composting of different types of solid wastes: Anaerobic digestion residue, pig manure and chicken manure. *Microbial Biotechnology*, 7(5), 424-433. <https://doi.org/10.1111/1751-7915.12131>
- Sun, R., Zhang, X. X., Guo, X., Wang, D., & Chu, H. (2015). Bacterial diversity in soils subjected to long-term chemical fertilisation can be more stably maintained with the addition of livestock manure than wheat straw. *Soil Biology and Biochemistry*, 88, 9-18. <https://doi.org/10.1016/j.soilbio.2015.05.007>
- Sweeten, J. M. (2008). *Composting manure and sludge* (E-479). Texas A&M AgriLife Extension Service.
- Takahashi, S., Ihara, H., & Karasawa, T. (2016). Compost in pellet form and compost moisture content affect phosphorus fractions of soil and compost. *Soil Science and Plant Nutrition*, 62(4), 399-404. <https://doi.org/10.1080/00380768.2016.1198680>
- Thompson, W., Leege, P., Millner, P., & Watson, M. (2002). *Test methods for the examination of composts and composting* (TMECC). The US Composting Council, US Government Printing Office.
- Truong, T. H. H., & Marschner, P. (2018). Respiration, available N and microbial biomass N in soil amended with mixes of organic materials differing in C/N and decomposition stage. *Geoderma*, 319, 167-174. <https://doi.org/10.1016/j.geoderma.2018.01.012>
- Vallini, G., Pera, A., Valdrighi, M., & Cecchi, F. (2002). Process constraints in source-collected vegetable waste composting. *Water Science and Technology*, 28(2), 93-100. <https://doi.org/10.2166/wst.1993.0110>
- Vergara, S. E., & Silver, W. L. (2019). Greenhouse gas emissions from windrow composting of organic wastes: Patterns and emissions factors. *Environmental Research Letters*, 14(12), Article 124006. <https://doi.org/10.1088/1748-9326/ab5262>
- Wang, K., Li, W., Li, X., & Ren, N. (2015). Spatial nitrifications of microbial processes during composting of swine, cow and chicken manure. *Scientific Reports*, 5(1), Article 14932. <https://doi.org/10.1038/srep14932>
- Wang, L., Li, Y., Prasher, S. O., Yan, B., Ou, Y., Cui, H., & Cui, Y. (2019). Organic matter, a critical factor to immobilise phosphorus, copper, and zinc during composting under various initial C/Ns. *Bioresource Technology*, 289, Article 121745. <https://doi.org/10.1016/j.biortech.2019.121745>
- Wang, X., Selvam, A., & Wong, J. W. C. (2016). Influence of lime on struvite formation and nitrogen conservation during food waste composting. *Bioresource Technology*, 217, 227-232. <https://doi.org/10.1016/j.biortech.2016.02.117>
- Warman, P. R. (1986). The effect of fertiliser, chicken manure and dairy manure on Timothy yield, tissue composition and soil fertility. *Agricultural Wastes*, 18(4), 289-298. [https://doi.org/10.1016/0141-4607\(86\)90074-0](https://doi.org/10.1016/0141-4607(86)90074-0)

- Wei, Y., Zhao, Y., Xi, B., Wei, Z., Li, X., & Cao, Z. (2015). Changes in phosphorus fractions during organic wastes composting from different sources. *Bioresource Technology*, 189, 349-356. <https://doi.org/10.1016/j.biortech.2015.04.031>
- Wen, X., Li, L., Sun, S., He, Q., & Tsai, F. S. (2019). The contribution of chicken products' export to economic growth: Evidence from China, the United States, and Brazil. *Sustainability*, 11(19), Article 5253. <https://doi.org/10.3390/su11195253>
- Wong, J. W. C., Wang, X., & Selvam, A. (2017). Improving compost quality by controlling nitrogen loss during composting. In H. H. Ngo, W. Guo, R. Y. Surampalli, & T. C. Zhang (Eds.), *Current developments in biotechnology and bioengineering: Solid waste management* (pp. 59-81). Elsevier. <https://doi.org/10.1016/B978-0-444-63664-5.00004-6>
- Yang, F., Li, G. X., Yang, Q. Y., & Luo, W. H. (2013). Effect of bulking agents on maturity and gaseous emissions during kitchen waste composting. *Chemosphere*, 93(7), 1393-1399. <https://doi.org/10.1016/j.chemosphere.2013.07.002>
- Zhang, D., Luo, W., Li, Y., Wang, G., & Li, G. (2018). Performance of co-composting sewage sludge and organic fraction of municipal solid waste at different proportions. *Bioresource Technology*, 250, 853-869. <https://doi.org/10.1016/j.biortech.2017.08.136>
- Zhang, Z., Liu, D., Qiao, Y., Li, S., Chen, Y., & Hu, C. (2021). Mitigation of carbon and nitrogen losses during pig manure composting: A meta-analysis. *Science of The Total Environment*, 783, Article 147103. <https://doi.org/10.1016/j.scitotenv.2021.147103>
- Zhou, J. M. (2017). The effect of different C/Ns on the composting of pig manure and edible fungus residue with rice bran. *Compost Science & Utilisation*, 25(2), 120-129. <https://doi.org/10.1080/1065657X.2016.1233081>
- Zhou, H. -B., Ma, C., Gao, D., Chen, T. B., Zheng, G. D., Chen, J., & Pan, T. H. (2014). Application of a recyclable plastic bulking agent for sewage sludge composting. *Bioresource Technology*, 152, 329-336. <https://doi.org/10.1016/j.biortech.2013.10.061>
- Zhou, Y., Xiao, R., Klammersteiner, T., Kong, X., Yan, B., Mihai, F. -C., Liu, T., Zhang, Z., & Awasthi, M. K. (2022). Recent trends and advances in composting and vermicomposting technologies: A review. *Bioresource Technology*, 360, Article 127591. <https://doi.org/10.1016/j.biortech.2022.127591>
- Zhu, N. (2007). Effect of low initial C/N ratio on aerobic composting of swine manure with rice straw. *Bioresource Technology*, 98(1), 9-13. <https://doi.org/10.1016/j.biortech.2005.12.003>

Effects of Soil Texture and Moisture on the Performance of EC-based Nutrient Sensor in Humid Tropical Mineral Soils

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ABSTRACT

An electrical conductivity (EC)-based nutrient sensor was tested on 37 Malaysian mineral soils, covering the texture and moisture range of humid tropical conditions, to estimate the apparent electrical conductivity (ECa), NO₃⁻, P, and K in real time. The sensor converts the nutrient ionic strength in the soil solution into electrical signals to estimate concentration. Against the 1:5 soil extract EC standard method, it estimated NO₃⁻ and K well but not P (Kling-Gupta Efficiency, KGE = 0.31), since orthophosphate ions contribute little to overall ionic strength. Soils were subjected to 13 moisture treatments ranging from 0% to 60% (w/w) to assess the EC-based nutrient sensor's performance. The study found that the sensor performed well (achieving KGE > 0.50, a mean absolute error <50%, and a bias error between -6% and 7%) in soils with clay content below 40% (v/v) and moisture levels above the saturation point (flooding conditions). However, results also indicated that the sensor underperformed in drier soils or clay content above 40% (achieving KGE < 0.39, a mean absolute error >48%, and a bias error between -26% and 11%) due to insufficient soil water for nutrient dissolution and reduced ionic mobility. These limitations must be considered when integrating EC-based nutrient sensors into IoT-based field data acquisition systems for real-time soil nutrient monitoring. While technological limitations remain, overcoming these challenges will allow this in-situ sensing framework to effectively characterise soil nutrient dynamics and establish the environmental thresholds necessary for precision, 'right-time' fertiliser management.

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INTRODUCTION

Nutrient content, particularly nitrogen (N), phosphorus (P), and potassium (K) - collectively known here in this paper as NPK - is important in supporting plant health and crop production (Žinete, 2023). To understand the soil nutrient status, soil nutrient analysis plays a pivotal role in sustainable agricultural development by ensuring balanced fertilisation, improving crop performance, and mitigating environmental degradation (Sahu et al., 2020). Traditional laboratory-based soil testing methods are the benchmark for accuracy, but they are time-consuming, expensive, and require specialised equipment and trained personnel. These limitations make them less practical for on-site or real-time decision-making, especially for resource-limited farmers.

Hence, innovative solutions are essential for delivering real-time, precise data on soil nutrient levels and addressing the limitations of traditional laboratory nutrient analysis (Islam et al., 2023). In recent years, the application of Internet of Things (IoT) technology for real-time soil nutrient monitoring has emerged (Reddy et al., 2023). The integration of EC-based nutrient sensors with IoT has enhanced resource efficiency by enabling real-time monitoring, reducing operational costs, and facilitating data-driven decisions through artificial intelligence in fertilisation systems. This has led to more sustainable and productive agriculture (Hossain et al., 2023; Nyakuri et al., 2022).

The EC-based nutrient sensor operates on the principle of apparent electrical conductivity (ECa), measuring bulk soil conductivity in real-time to estimate nutrient content (Corwin & Yemoto, 2020). Nevertheless, ECa is influenced by soil texture. Clay soils hold more water gravimetrically than sandy soils; therefore, the former soils have greater water-holding capacity (Rout & Arulmozhiselvan, 2019). That difference affects ECa, and measurements lose reliability under fluctuating moisture (Costa et al., 2014), complicating nutrient estimation in heterogeneous soils.

The ECa-nutrient relationship is inconsistent across soil types, spatial gradients, and cropping systems, with location-specific factors adding further noise (Heiniger et al., 2003; Mazur et al., 2022; Omonode & Vyn, 2006). Fertiliser applications temporarily raise EC, but the increase does not consistently correspond to nutrient availability across soils (Othaman et al., 2021). EC-based sensors have advanced, allowing for real-time nutrient monitoring, but the diverse physical and chemical properties of soils prevent them from fully capturing soil-nutrient interactions, producing errors under variable field conditions.

Although Dimkpa et al. (2017) found few independent assessments of rapid nutrient testing technologies, sensor accuracy remains under-documented in the literature. Most studies have integrated EC sensors into IoT frameworks for real-time monitoring without validating accuracy across soil textures and moisture levels. Soil texture, moisture content, chemical composition, and microbial communities each shift readings independently, making direct nutrient detection unreliable (Nadporozhskaya et al., 2022). This limits

EC-based sensors in heterogeneous tropical agricultural soils, which are predominantly weathered and acidic and cover about 43% of Earth's land area (Nottingham et al., 2022). These sensors warrant further investigation, though their reliability remains constrained by soil heterogeneity and dynamic field conditions.

This study evaluated an EC-based nutrient sensor across varied soil textures and moisture conditions in selected tropical mineral soils. The sensor design is specific, but the findings address the broader relationships among soil EC, moisture, and NO_3^- , P, and K captured by EC-based devices. Performance was assessed through simple linear regression models validated against laboratory data using three metrics: Kling-Gupta Efficiency (KGE), Normalised Mean Absolute Error (NMAE), and Normalised Mean Bias Error (NMBE). Understanding how the sensor performs across soil conditions will inform strategies for reliable soil fertility monitoring.

This study focuses on selected Peninsular Malaysian mineral soils, which are taken as representative of humid tropical soils. These soils are characterised by intense weathering, significant nutrient leaching, acidity ($\text{pH} < 5$), low cation exchange capacity ($\text{CEC} < 20 \text{ cmol/kg}$), low total phosphorus ($\leq 24\%$), high exchangeable aluminium in some areas, and a predominance of Oxisols with a silt-to-clay ratio of less than 0.15. Oxisols cover approximately 23% of the Earth's land area (Dion, 2010; Nottingham et al., 2022).

Unlike previous studies that primarily focus on IoT frameworks, this study uniquely investigates the responses of an EC-based nutrient sensor in determining nutrients (EC, NPK) under varying soil moisture and texture conditions in Malaysian mineral soils, which are representative of humid tropical environments. However, due to the specificity of humid tropical soil properties, the findings should be interpreted with caution when applied to other soil types beyond this region.

EC-based sensors offer a faster alternative to laboratory testing for real-time soil fertility monitoring, but how soil texture and moisture affect bulk conductivity and NPK estimation in humid tropical mineral soils is still not well characterised. Consequently, this study aimed to evaluate how both variables influence EC-based sensor performance for estimating these macronutrients, hypothesising that texture and moisture alter ECa by governing water availability and ion mobility, which affects estimation accuracy.

MATERIALS AND METHODS

Soil Sampling and Laboratory Soil Assays

To capture a wide range of soil physical and chemical properties—particularly soil texture variations—at selected field sites in Malaysia representing tropical mineral soils, a point sampling approach was employed. Individual soil samples were collected from distinct locations, each representing different soil types, land uses, and agroecological conditions (Figure 1). This approach captured diverse soil characteristics rather than spatial variability

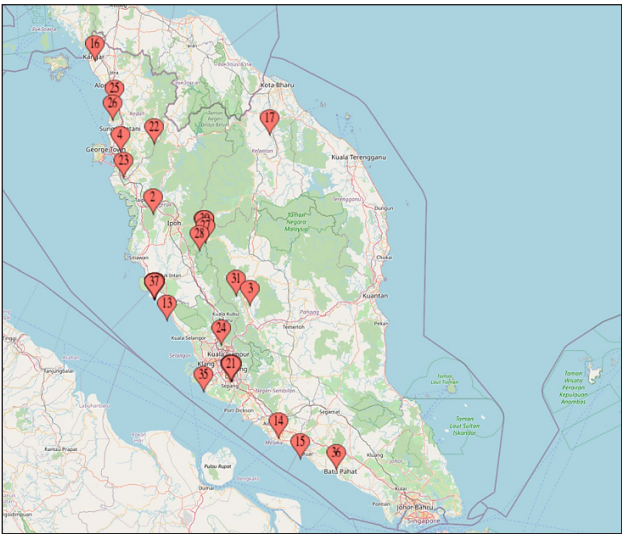


Figure 1. Spatial distribution of the 37 soil sampling locations across Peninsular Malaysia, representing diverse soil textures, land uses, and agroecological zones

within a single field. Sampling was carried out in 2024 across locations with a humid tropical climate, high temperatures, and variable rainfall. Thirty-seven topsoil samples (0–10 cm) were selected using a point sampling approach, spanning a regional gradient of soil textures, parent materials, and crop management histories. This layer is the primary zone of root activity and fertiliser application. Samples were air-dried, ground, and sieved through a 2-mm mesh. Air-dried soil forms hard clumps that can damage the probe, so crushing and sieving were necessary before sensor insertion. Laboratory assays characterised soil chemical and physical properties (Table 1 and Table 2). All laboratory characterisation and sensor measurements were conducted in triplicate.

For soil chemical properties, the following parameters were measured: pH, EC, total nitrogen (TN), NH_4^+ , NO_3^- , available P, and exchangeable K. These analyses followed the protocols outlined by Reeuwijk (2002), Gavlak et al. (2005), and Pansu et al. (2006).

The pH and EC of the suspension were measured using a Hanna Edge pH meter and a Hanna HI2300 EC meter (Hanna Instruments, USA). NH_4^+ and NO_3^- concentrations in the filtrate were analysed using a SEAL AA500 AutoAnalyzer (SEAL Analytical, Germany). Available P was measured using a Series 1000 UV/Visible Spectrophotometer (Cecil Instruments, UK) at 882 nm. Exchangeable K was determined using a PinAAcle 900T Atomic Absorption Spectrometer (PerkinElmer, USA).

Soil physical properties, including soil texture, were determined according to the methods described by Gee & Bauder (1986), and soil water retention was measured using the procedure outlined by Klute (1986).

Table 1
Chemical properties and dataset categorisation of soil samples

Sample	TN	NH ₄ ⁺	NO ₃ ⁻	Exchangeable		pH	EC μS cm ⁻¹	Dataset Purpose
	%			mg kg ⁻¹	K			
1	0.13	20.2	1.4	92.9	1468.4	6.2	156.5	Calibration
2	0.12	36.2	8.1	10.6	400.8	3.9	72.4	
3	0.14	29.9	2.0	15.4	452.3	5.8	55.1	
4	0.15	29.9	24.5	10.9	540.6	4.5	91.0	
5	0.14	30.8	22.7	144.1	1495.3	5.4	64.4	
6	0.10	32.7	8.6	247.4	448.8	4.8	49.2	
7	0.05	29.2	12.7	466.8	465.8	5.1	62.9	
8	0.10	22.4	4.9	54.4	647.1	4.7	56.8	
9	0.18	28.9	2.0	54.4	740.0	5.8	23.9	
10	0.19	38.2	8.8	133.5	946.0	5.1	31.1	
11	0.22	37.9	30.0	3057.0	3192.4	7.0	200.9	
12	0.04	0.2	N/A	9.6	691.2	5.9	17.5	
13	0.10	25.9	93.4	220.6	5311.0	6.4	460.3	
14	0.13	36.1	66.0	1466.2	3528.7	7.2	188.8	
15	0.15	28.2	22.7	28.7	464.4	4.8	20.8	
16	0.20	30.3	1.1	136.9	1532.1	4.7	44.6	
17	0.15	25.1	5.9	255.1	1716.1	6.6	146.0	
18	0.19	24.3	6.6	221.7	2483.6	7.1	93.4	
19	0.19	32.7	7.6	354.5	5340.1	7.4	147.2	
20	0.40	18.9	6.4	363.1	1179.4	4.5	283.9	
21	0.12	42.1	18.8	94.0	438.1	4.7	62.2	
22	0.08	20.4	0.6	105.1	491.6	6.8	78.1	
23	0.18	20.4	8.3	261.0	1617.4	7.2	91.3	
24	0.09	22.8	0.3	18.9	869.6	4.4	44.3	

Table 1 (continued)

Sample	TN	NH ₄ ⁺	NO ₃ ⁻	mg kg ⁻¹		Exchangeable K	pH	EC	Dataset Purpose
	%			Available P				µS cm ⁻¹	
25	0.06	23.5	N/A	29.9	999.0	6.0	58.3		
26	0.08	20.0	20.9	48.8	775.9	5.8	106.5		
27	0.12	34.1	10.8	21.2	709.3	5.8	84.1		Validation
28	0.20	40.0	22.6	77.6	487.6	4.5	50.7		
29	0.24	34.3	8.5	45.7	799.0	5.0	53.9		
30	0.14	23.6	16.0	28.9	456.5	5.7	77.5		
31	0.14	28.9	70.0	561.4	5897.0	6.9	401.0		
32	0.18	28.0	6.6	228.3	1773.0	6.2	103.1		
33	0.21	24.4	18.3	400.9	3335.1	6.7	245.9		
34	0.08	19.6	8.2	234.4	513.6	7.2	87.7		
35	0.12	31.3	34.7	20.2	1248.6	4.7	130.0		
36	0.05	18.9	0.5	47.5	388.6	6.6	75.6		
37	0.09	21.9	24.6	171.2	1486.7	6.4	155.2		

Note. N/A: Not available

Table 2
Physical properties, sampling locations, and land use of soil samples

Sample	SP		FC %	PWP	USDA Texture (%clay, %sand)	Location °N, °E	Land Use
1	44	29	22		C (59, 13)	3.656278, 101.008444	Cocoa (<i>Theobroma cacao</i>)
2	37	20	14		SCL (34, 54)	3.001083, 101.724556	Pine (<i>Pinus spp.</i>)
3	38	22	18		C (59, 35)	2.988389, 101.724500	Oil palm (<i>Elaeis guineensis</i>)
4	39	22	17		C (57, 38)	2.987972, 101.728000	Oil palm
5	34	21	15		CL (39, 30)	5.602833, 100.861028	Vegetables
6	26	6	4		LS (8, 87)	5.233528, 100.527111	Vegetables
7	25	8	4		LS (6, 88)	3.381000, 101.608500	Vegetables
8	34	20	14		SCL (34, 46)	2.355306, 102.245722	Cocoa
9	41	27	20		C (52, 11)	6.026056, 100.421083	Paddy (<i>Oryza sativa</i>)
10	38	22	11		SL (17, 56)	5.870167, 100.403528	Paddy
11	41	21	14		SL (14, 61)	4.511222, 101.433611	Vegetables
12	41	27	21		C (44, 43)	4.425639, 101.368278	Open land (Cleared Forest)
13	40	25	19		C (42, 35)	4.589389, 101.419972	Vegetables
14	39	23	15		SL (19, 56)	4.590750, 101.422778	Vegetables
15	35	22	14		L (24, 40)	3.931667, 101.774472	Durian (<i>Durio zibethinus</i>)
16	46	34	27		C (66, 2)	3.895806, 100.868222	Cocoa
17	45	32	24		SiC (51, 8)	3.897778, 100.869500	Cocoa
18	47	33	26		C (67, 1)	3.900222, 100.872222	Cocoa
19	45	32	24		C (62, 5)	3.899167, 100.869722	Cocoa
20	47	28	18		CL (28, 40)	2.867944, 101.414694	Oil palm

Table 2 (continued)

Sample	SP		FC		PWP		USDA Texture (%clay, %sand)	Location °N, °E	Land Use
				%					
21	35		20		10		SL (16, 63)	2.000111, 102.889444	Water discharge from an Arowana (<i>Scleropages formosus</i>) fishpond area
22	28		8		4		LS (6, 88)	3.895778, 100.869167	Cocoa
23	40		27		19		C (48, 20)	3.895806, 100.868222	Cocoa
24	40		27		19		C (56, 10)	2.123222, 102.492694	Cocoa
25	30		15		7		L (18, 52)	6.528056, 100.203528	Cocoa
26	35		17		6		SL (14, 72)	5.707917, 102.149333	Cocoa
27	37		18		13		SC (40, 51)	2.982611, 101.709556	Coconut (Cocos nucifera)
28	37		18		12		SCL (31, 61)	2.982722, 101.724056	Rubber (<i>Hevea brasiliensis</i>)
29	43		28		23		S (68, 23)	2.982472, 101.722417	Coconut
30	32		11		7		SL (16, 78)	2.988750, 101.723194	Oil palm
31	39		25		18		CL (30, 43)	4.590222, 101.421917	Vegetables
32	44		32		25		SiC (58, 1)	3.896556, 100.864778	Cocoa
33	44		32		24		C (65, 1)	3.910000, 100.871417	Cocoa
34	28		10		5		LS (9, 86)	3.893778, 100.864861	Cocoa
35	38		21		16		SC (48, 47)	4.824278, 100.857556	Cocoa
36	29		12		5		SL (16, 65)	3.820083, 101.932194	Cocoa
37	34		20		12		CL (29, 43)	5.519472, 100.495333	Cocoa

Note. SP: Saturated point (wet basis). FC: Field capacity (wet basis). PWP: Permanent wilting point (wet basis). C: Clay. CL: Clay loam. L: Loam. LS: loamy sand. SCL: Sandy clay loam. SL: sandy loam. SC: Sandy loam. SiC: Silty clay

Relationship between EC and NPK in Laboratory Analysis

The soil EC and NPK data were obtained through laboratory analysis and further investigated to determine their relationship. A total of 37 soil samples were used in this study. Pearson correlation analysis was first conducted to assess the strength and direction of the relationships between EC and the concentrations of TN, NH_4^+ , NO_3^- , P, and K across all 37 soil samples. A minimum correlation threshold of $r \geq 0.60$ was set as a prerequisite for subsequent regression development. Setting this threshold at this level will determine EC's predictive power for these nutrients.

The 37 soil samples were randomly divided into calibration and validation groups in a 70:30 ratio, respectively, where the calibration group, comprising 26 soil samples, was utilised to construct simple linear regression models to predict nutrient concentrations based on laboratory EC values. 95% confidence intervals were calculated for all regression coefficients to establish parameter precision. The validation group, comprising 11 samples, was for independent validation. These allocations are detailed in Table 1 under the 'Dataset Category', with 26 samples designated for calibration and 11 for validation using R statistical software (version 4.3.1).

Validation of the models' prediction performance was assessed using three statistical metrics: KGE, NMAE, and NMBE. The last two metrics were selected to assess overall model accuracy and model bias, respectively, whereas KGE was chosen because it simultaneously assesses a model's performance based on three criteria: correlation, bias, and variability between observed and predicted values. KGE ranges from $-\infty$ to 1, with a value of 1 indicating perfect agreement. NMAE measures the average prediction error normalised to the observed range, allowing for an evaluation of how closely the predictions align with observed values, where values range from 0 to $+\infty$, with 0 indicating perfect agreement. NMBE quantifies model bias, offering insight into whether the predictions systematically overestimate or underestimate observations, with values ranging from $-\infty$ to $+\infty$ and 0 indicating no bias (Gupta et al., 2009)

$$KGE = 1 - \sqrt{(r - 1)^2 + (\beta - 1)^2 + (\gamma - 1)^2} \quad [1]$$

where r is the correlation coefficient between predicted and observed values; β is the bias ratio, defined as μ_p/μ_o , where μ_p is the mean predicted value, and μ_o is the mean observed value (both expressed in the same unit); and $\gamma = (\sigma_o/\mu_o)/(\sigma_p/\mu_p)$ the variability ratio, which compares the observed coefficient of variation (σ_o/μ_o) to the predicted coefficient of variation (σ_p/μ_p).

$$NMAE = \frac{\sum_{i=1}^n |P_i - O_i|}{n \times \bar{O}} \quad [2]$$

where P_i represents the predicted values; O_i represents the observed values; $\bar{O}$ represents the mean of all observed values, respectively (all in the same unit); n represents the total number of observations.

$$NMBE = \frac{\sum_{i=1}^n (P_i - O_i)}{n \times \bar{O}} \quad [3]$$

These three metrics (Equations 1 - 3) were chosen for their distinct strengths: KGE combines correlation, bias, and variability into a single score for overall model fit; NMAE measures overall model prediction errors; and NMBE measures model bias, revealing whether the model consistently over- or under-predicts.

Together, these metrics assess both the EC-nutrient relationship and the practical reliability of the regression models for estimating nutrient concentrations from EC measurements.

Evaluation of an EC-based Electronic Nutrient Sensor under Various Soil Moisture Conditions

The EC-based electronic nutrient sensor, manufactured by JXCT® (Shandong Jingxunchangtong Electronic Technology Co., Ltd., China), has two stainless steel electrodes that measure soil NPK, temperature, moisture, pH, and EC (Figure 2). The probes are IP68-rated, use an RS485 output signal, and operate at 9-24 V DC. Data are displayed on the sensor monitor.

Soil moisture was controlled in a laboratory to isolate its effect on sensor performance from field variables such as evaporation under direct sunlight.

Moisture levels were adjusted from 0% to 60% (w/w) at 5% intervals. The 60% ceiling was set to exceed the saturation point (wet basis) of each soil, enabling evaluation under



Figure 2. Stainless steel soil NPK, temperature, moisture, EC and pH electrical probes

fully saturated conditions. For a 250 g subsample, 12.5 g of water (5% of 250 g) was added per step. After each addition, subsamples were equilibrated for one day to allow uniform water distribution before sensor measurements.

At each moisture level, sensor readings were taken after 1 min for all three subsamples of each soil (Figure 3). Soil moisture (v/v) was measured immediately after using a FieldScout TDR 100 (Spectrum Technologies, Inc., USA). This sensor was calibrated before each use and served as a moisture reference, given its established use in soil moisture determination. These readings were compared against the saturation point (SP, wet basis) measured for each soil using the pressure plate extractor method. Full saturation was confirmed when TDR readings stabilised at each soil’s SP threshold.

Optimisation and Evaluation of EC-Based Electronic Nutrient Sensor

A total of 1,443 sensor readings were collected by monitoring the 37 soil samples across sequential moisture content adjustments, ranging from 0% to 60% (w/w) at 5% intervals. To evaluate sensor performance against laboratory values across varying moisture matrices,



Figure 3. Soil parameters determination using EC-based electronic nutrient sensor (Shandong Jingxunchangtong Electronic Technology Co., Ltd., China)

the dataset was partitioned strictly by soil sample, matching the independent 26-calibration and 11-validation sample groupings detailed in Table 1, was applied to categorise the data into calibration and validation sets.

To optimise the performance of the electronic in-situ nutrient sensor, data were filtered based on soil moisture conditions measured using the FieldScout TDR 100 soil moisture meter and soil texture. Specifically, soil texture was filtered by clay percentage to ensure the calibration models accurately accounted for the physical properties influencing nutrient estimation. A simple linear regression modelling approach was employed to develop predictive models linking the electronic in-situ sensor values (EC, NO_3^- , P, and K) to laboratory-determined soil nutrient concentrations. Sensor performance was evaluated using three statistical metrics—KGE, NMAE, and NMBE—to ensure accuracy and reliability across diverse soil conditions.

RESULTS AND DISCUSSIONS

Relationship between EC and NPK in Laboratory Analysis

EC and forms of N

TN is commonly analysed in the laboratory to understand the combined amount of organic and inorganic N in the soil. While NH_4^+ and NO_3^- represent the inorganic forms of N that are directly available for plant uptake and are the primary sources derived from nitrogen fertilisers.

From Figure 4 (a) and (b), no significant relationships were observed between EC and TN or NH_4^+ from laboratory analysis. This lack of correlation can be attributed to NH_4^+ , which is positively charged and strongly adsorbs onto negatively charged soil particles, such as clay minerals. This adsorption reduces its mobility in the soil (Yu et al., 2023). Moreover, soil TN comprises organic forms like proteinaceous materials and heterocyclic compounds, as well as inorganic forms such as ammonia. Many of these N sources are not part of the soil solution, thus not contributing directly to ionic conductivity (Schulten & Schnitzer, 1997). As a result, laboratory EC measurements were less effective in predicting TN or NH_4^+ concentrations due to lower ionic conductivity strength.

In contrast, Figure 4 (c) shows a strong positive correlation between EC and NO_3^- concentrations ($r = 0.75$), indicating that higher EC levels correspond to increased NO_3^- content in the soil. This relationship is attributed to NO_3^- is high mobility in water and a significant contribution to soil ionic conductivity. Analysis of 26 soil samples with varying land uses and chemical and physical properties revealed that NO_3^- concentrations can be estimated as 16% of EC values (Figure 4 (d)).

According to Thiemiig et al. (2013), KGE values can be classified into performance levels: poor performance ($0.50 > \text{KGE} > 0$), intermediate performance ($0.75 > \text{KGE} > 0.50$), and good performance ($\text{KGE} > 0.75$). Based on this classification, the NO_3^- model

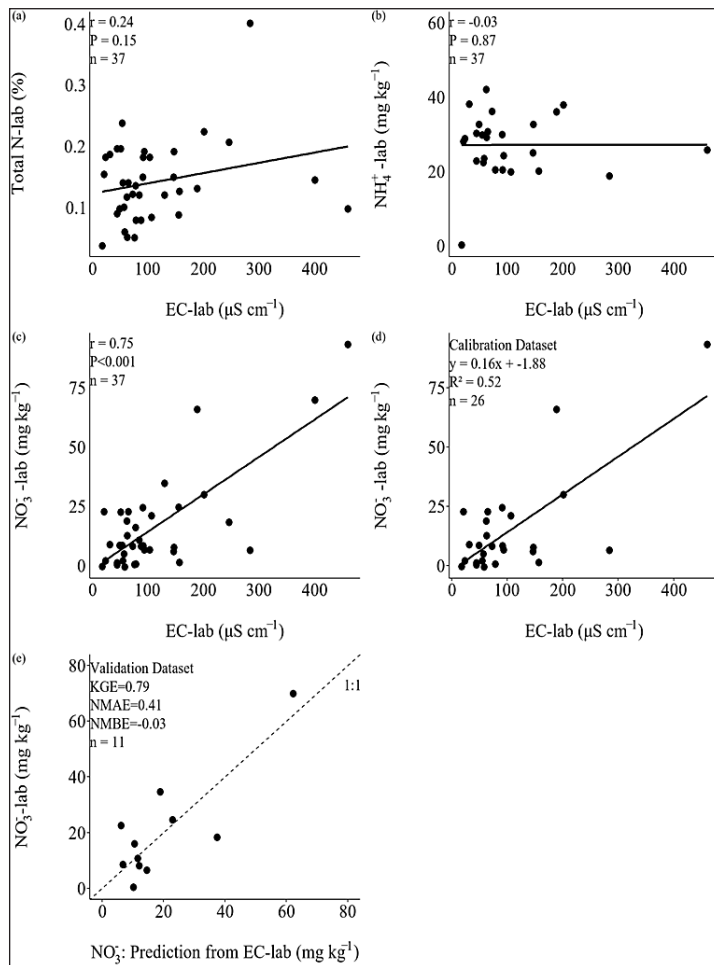


Figure 4. Relationships between laboratory-measured EC and total nitrogen (TN) concentrations (a), EC and NH_4^+ concentrations (b), and EC and NO_3^- concentrations (c); (d) NO_3^- prediction model; and (e) NO_3^- prediction using 1:5 EC values

demonstrates good performance ($\text{KGE} = 0.79$) (Figure 4 (e)), indicating its reliability for predicting nitrate concentrations by EC with moderate error ($\text{NMAE} = 0.41$) and minimal bias ($\text{NMBE} = -0.03$). These findings are consistent with previous studies, highlighting the potential of EC-based regression models, such as those using TDR sensors and the Rhoades model, for continuous monitoring of soil nitrate levels in Andisol fields (Miyamoto et al., 2015).

EC and P

A moderate positive correlation ($r = 0.60$) was observed between EC and P in laboratory analysis after excluding two outlier samples with extremely high phosphorus concentrations

($>1000 \text{ mg kg}^{-1}$) from the 37 soil samples (Figure 5 (a)). These outliers were identified and removed based on the interquartile range (IQR) method, as the extreme values were attributed to localised over-fertilisation of phosphorus. However, Figure 5 (c) indicate that the P prediction model based on EC had poor performance ($\text{KGE}=0.31$) and 63% mean absolute error. Orthophosphate ions (plant-available P) have lower ionic conductivity in soil solution than nitrate and high natural variability. P availability is governed by pH, mineralogy, microbial solubilization, and organic matter interactions. Iron oxyhydroxides, struvite, vivianite, and organic matter adsorb and precipitate P, while certain bacteria enhance availability by producing organic acids that solubilise bound P, particularly in acidic soils (Ahmed et al., 2023; Azam et al., 2019; Herndon et al., 2020; Tamad et al., 2020). This variability across soils makes P concentration difficult to estimate from EC measurements alone.

EC and K

K correlated positively with EC ($r=0.64$, Figure 6a) across selected Malaysian agricultural soils. This is expected because cations, including potassium, calcium, and magnesium, drive soil electrical conductivity, and their concentration and mobility in the soil solution determine EC readings (Rhoades et al., 1989).

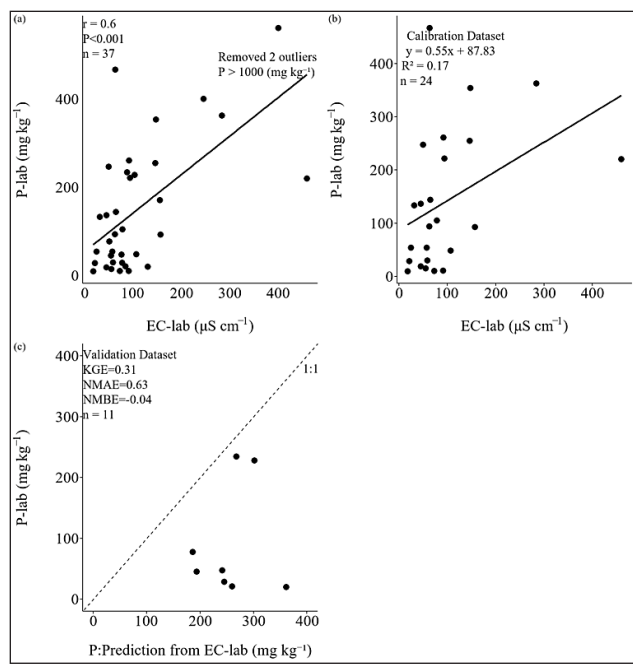


Figure 5. (a) Relationships between laboratory-measured EC and P concentrations; (b) P prediction model; and (c) P prediction using 1:5 EC values

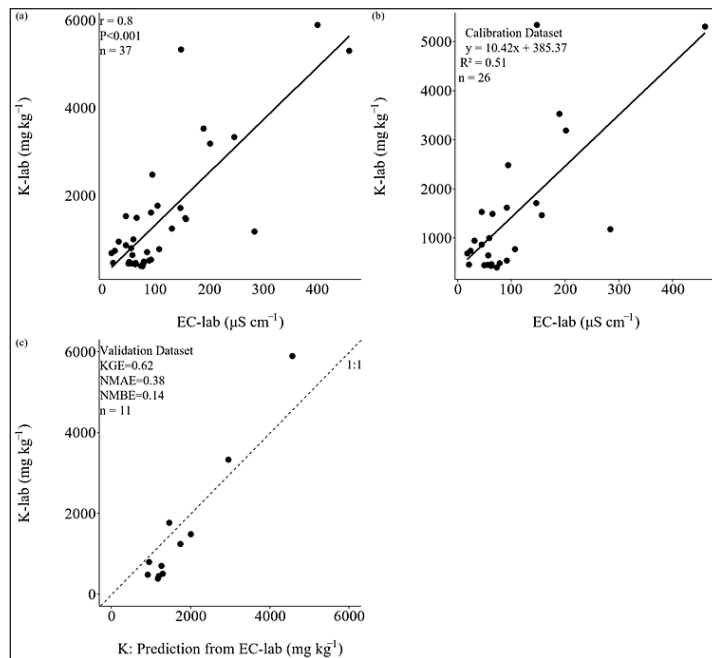


Figure 6. (a) Relationships between laboratory-measured EC and K concentrations; (b) K prediction model; and (c) K prediction using 1:5 EC values

The K prediction model was overall accurate ($KGE = 0.62$, Figure 6c), capturing the trends in exchangeable K across the sampled soils. The 1:5 soil-to-water EC extraction method estimated K concentrations with sufficient accuracy for rapid nutrient determination.

Optimisation and Evaluation of an Electronic EC-based Nutrient Sensor

The electronic nutrient sensor relies on ECa to estimate soil NPK concentrations, with soil moisture content significantly affecting sensor readings. Figure 7 showed that readings of the sensor for ECa, NO_3^- , P, and K spiked then stabilised once soil moisture exceeded the saturation point in loamy sand. This moisture dependence makes the sensor unsuitable for dry soil conditions.

Moreover, this study revealed that soil texture also affected the sensor's performance to optimise its functionality. The sensor was suitable for applications where clay content was below 30%, with soil moisture (v/v) ranging from 40% to 55% for NO_3^- . While the sensor was effective with clay content below 40%, and soil moisture (v/v) ranging from 40% to 60% for EC, P, and K determination (Table 3). The sensor's best performance was at 40-60% (v/v) moisture because of greater dissolved nutrients and higher ionic conductivity in the soil solution. Higher moisture facilitates mobile nutrient transport (Bauke et al., 2022), and in low-clay soils, NO_3^- , P, and K become more available, increasing ionic conductivity and improving nutrient estimation. At lower moisture levels, nutrient dissolution and mobility

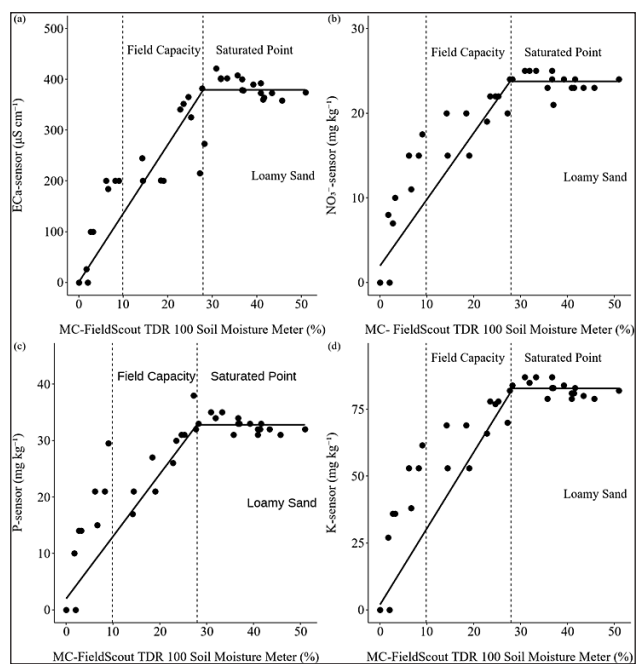


Figure 7. Performance of the EC-based nutrient sensor under varying soil moisture in loamy sand: a) EC response, b) NO₃⁻ response, c) P response and d) K: response

Table 3
Criteria for EC-based electronic nutrient sensor

Parameter	MC reading from FieldScout Soil Moisture Meter (%) (v/v)	Clay (%)
EC	40<MC<60	<35
NO ₃ ⁻	40<MC<55	<35
P	40<MC<60	<40
K	40<MC<60	<40

Note. MC: soil moisture content. The EC-based electronic nutrient sensor is valid for P concentrations below 1000 mg kg⁻¹

are limited, resulting in less reliable readings. Conversely, excessive moisture beyond the saturation point can also dilute nutrient concentrations, reduce sensor accuracy, and negatively affect the soil environment, such as nutrient leaching.

The EC-based electronic nutrient sensor readings required calibration to achieve accurate results, as outlined in the criteria in Table 3. The calibration models for EC, NO₃⁻, P, and K demonstrated intermediate performance, achieving KGE > 0.50, NMAE < 0.50, and -0.06 ≤ NMBE ≤ 0.07 (Figure 8). While these metrics indicate the potential of the models for rapid nutrient estimation, they also highlight room for improvement, especially for high-precision applications.

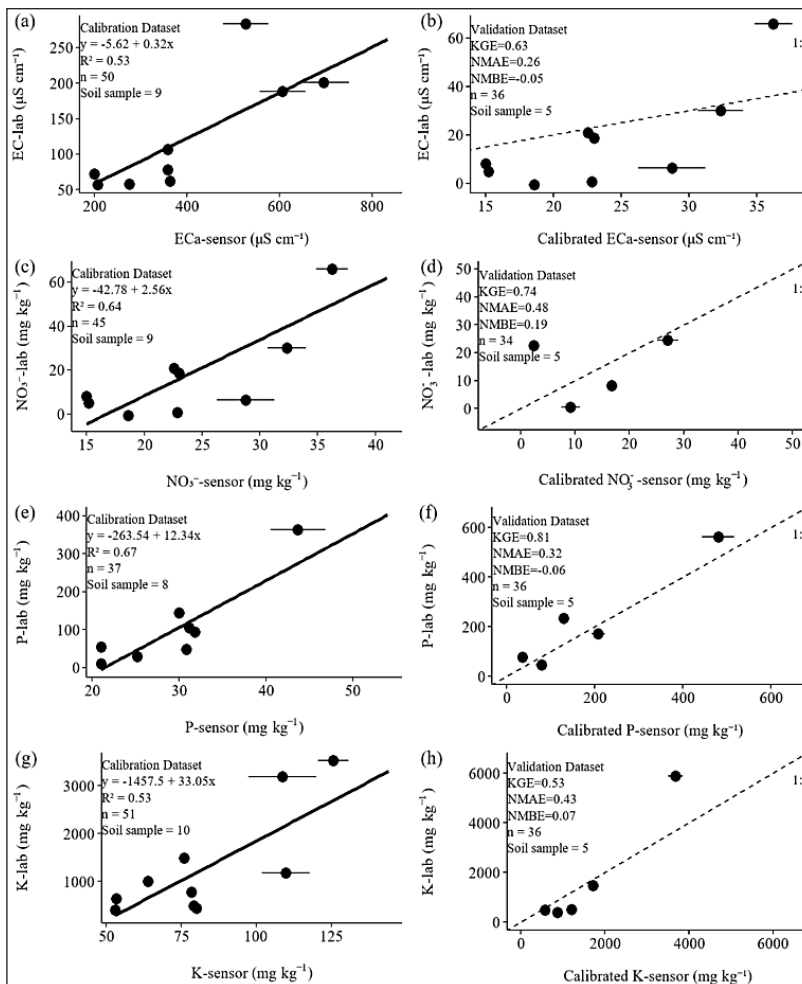


Figure 8. Calibration analysis and validation of the EC-based electronic nutrient sensor against laboratory-measured values, with criteria outlined in Table 3. Error bars represent the mean ($\pm$ standard error). (a) EC calibration model; (b) EC validation; (c) NO_3^- calibration model; (d) NO_3^- validation; (e) P calibration model; (f) P validation; (g) K calibration model; and (h) K validation

Despite the applicability of these calibration models, the deployment of EC-based electronic nutrient sensors presents two major challenges: soil type specificity and the necessity for high soil moisture levels. When the soil conditions have a clay percentage higher than 30-40% and soil moisture within the range of 10-40% (v/v) (field capacity range) as measured by the FieldScout Soil Moisture Meter, the electronic nutrient sensor performs poorly in estimating soil nutrients (Figures 9 and 10). This is because sandy and silty soils are negatively correlated with CEC, whereas clayey soils have a positive impact on CEC (Obalum et al., 2013), allowing them to retain more nutrients, while CEC is often

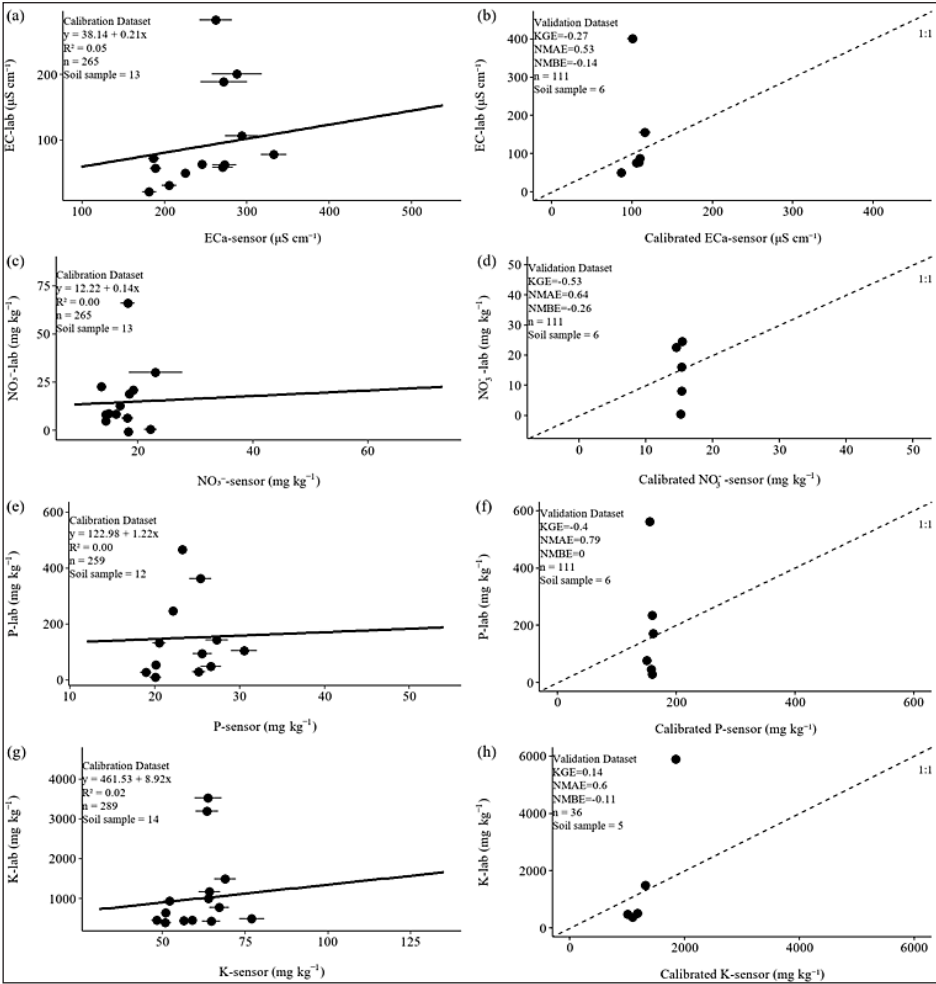


Figure 9. Calibration and validation analysis of the EC-based electronic nutrient sensor against laboratory-measured values, with moisture content within the field capacity range (10-40% v/v) as measured using the FieldScout Soil Moisture Meter. Error bars represent the mean ($\pm$ standard error). (a) EC calibration model; (b) EC validation; (c) NO_3^- calibration model; (d) NO_3^- validation; (e) P calibration model; (f) P validation; (g) K calibration model; and (h) K validation

correlated with ECa (Zhao et al., 2022). Furthermore, the sensor does not perform optimally at field capacity due to the lower availability of dissolved ions in soil water to convert electrical signals. Soils with clay content above 40% are unsuitable for this sensor. Stable readings also require 40-60% (v/v) moisture, which exceeds the saturation point of most soils. Sustained saturation causes flooding, creating anaerobic conditions that inhibit root function and can trigger root rot (Chadha et al., 2019). Field deployment must therefore account for clay content and moisture management.

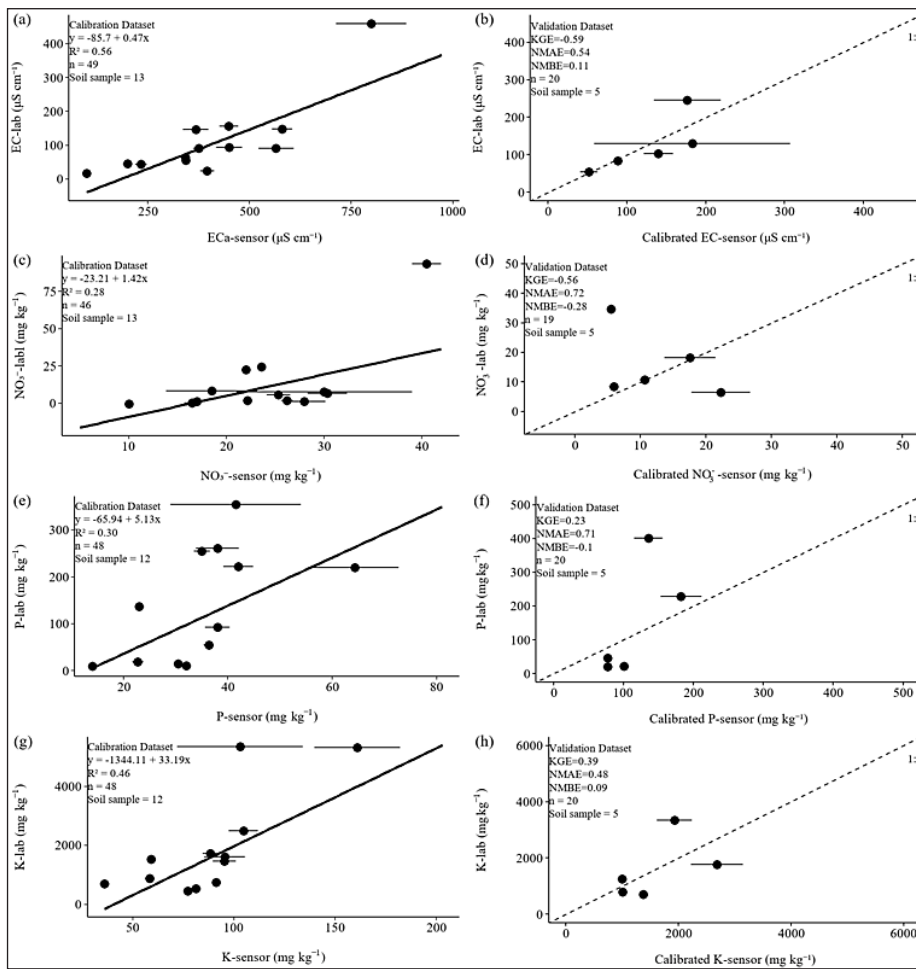


Figure 10. Calibration analysis and validation of the EC-based electronic nutrient sensor against laboratory-measured values, with clay percentage criteria higher than those outlined in Table 3. Error bars represent the mean ($\pm$ standard error). (a) EC calibration model; (b) EC validation; (c) NO_3^- calibration model; (d) NO_3^- validation; (e) P calibration model; (f) P validation; (g) K calibration model; and (h) K validation

CONCLUSION

This study demonstrates that electronic EC-based sensors provide a viable means of estimating NO_3^- and K levels in humid tropical mineral soils, though their efficacy is strictly influenced by soil physical properties. The 1:5 EC extraction method reliably estimated NO_3^- and K but not P, where the low ionic contribution of orthophosphate produced a poor model fit ($KGE = 0.31$). The sensor is also restricted to soils below 40% clay; above that threshold, predictive accuracy drops. Performance peaks at near-saturated moisture, though that range risks anaerobic conditions in the root zone. Implementing EC-based sensors in tropical agriculture will require calibration models that account for high clay content and variable moisture regimes.

IMPLICATION OF THE STUDY

Results from this study inform sensor integration for precision agriculture in tropical regions. EC-based sensing works for N and K but not P, which shapes nutrient management protocols accordingly. Texture-specific calibration is necessary: the 40% clay threshold marks where sensor accuracy breaks down. The moisture dependency suggests future designs should incorporate secondary transducers or compensatory logic to remain functional at field capacity. These constraints should guide IoT-based monitoring deployment and ensure data-driven fertilisation accounts for the electrochemical and physical properties of humid tropical mineral soils.

LIMITATION AND RECOMMENDATION FOR FUTURE RESEARCH

The small sample size may limit the generalizability of findings across the full range of humid tropical mineral soils. The results confirm that EC-based sensors face substantial limitations in estimating NO_3^- , P, and K in heterogeneous tropical soils, despite their widespread integration into IoT platforms for real-time monitoring. Future research should draw on a wider range of soils and incorporate geospatial approaches, including fuzzy logic, geostatistical methods, and GIS techniques (Seyedmohammadi et al., 2019), to improve calibration and site-specific nutrient estimation. Sensor design should also target sensitivity and reliability at field capacity, which better represents actual growing conditions. In regions with high soil variability, precision agriculture adoption should proceed cautiously, with priority given to localised calibration.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study, which include EC-based nutrient sensor measurements for 37 soil samples subjected to 13 moisture treatments, are openly available in [GitHub Repository] at https://github.com/Melissateng97/Thesis_data/

REFERENCES

- Ahmed, A. A., Leinweber, P., & Kühn, O. (2023). Advances in understanding the phosphate binding in soil: A computational chemistry perspective. *Science of The Total Environment*, 887, Article 163692. <https://doi.org/10.26434/chemrxiv-2022-lq2j3>
- Azam, H. M., Alam, S. T., Hasan, M., Yameogo, D. D. S., Kannan, A. D., Rahman, A., & Kwon, M. J. (2019). Phosphorous in the environment: Characteristics with distribution and effects, removal mechanisms,

- treatment technologies, and factors affecting recovery as minerals in natural and engineered systems. *Environmental Science and Pollution Research*, 26(20), 20183-20207. <https://doi.org/10.1007/s11356-019-04732-y>
- Bauke, S. L., Amelung, W., Bol, R., Brandt, L., Brüggemann, N., Kandeler, E., Meyer, N., Or, D., Schnepf, A., Schloter, M., Schulz, S., Siebers, N., von Sperber, C., & Vereecken, H. (2022). Soil water status shapes nutrient cycling in agroecosystems from micrometer to landscape scales. *Journal of Plant Nutrition and Soil Science*, 185(6), 773-792. <https://doi.org/10.1002/jpln.202200357>
- Chadha, A., Florentine, S. K., Chauhan, B. S., Long, B., & Jayasundera, M. (2019). Influence of soil moisture regimes on growth, photosynthetic capacity, leaf biochemistry and reproductive capabilities of the invasive agronomic weed; *Lactuca serriola*. *PLOS ONE*, 14(6), Article e0218191. <https://doi.org/10.1371/journal.pone.0218191>
- Corwin, D. L., & Yemoto, K. (2020). Salinity: Electrical conductivity and total dissolved solids. *Soil Science Society of America Journal*, 84(5), 1442-1461. <https://doi.org/10.1002/saj2.20154>
- Costa, M. M., Queiroz, D. M. D., Pinto, F. D. A. D. C., Reis, E. F. D., & Santos, N. T. (2014). Moisture content effect in the relationship between apparent electrical conductivity and soil attributes. *Acta Scientiarum. Agronomy*, 36(4), 395-401. <https://doi.org/10.4025/actasciagron.v36i4.18342>
- Dimkpa, C., Bindraban, P., McLean, J. E., Gatere, L., Singh, U., & Hellums, D. (2017). Methods for rapid testing of plant and soil nutrients. In E. Lichtfouse (Ed.), *Sustainable agriculture reviews* (Vol. 25, pp. 1-43). Springer International Publishing. https://doi.org/10.1007/978-3-319-58679-3_1
- Dion, P. (Ed.). (2010). *Soil biology and agriculture in the tropics* (Vol. 21). Springer Berlin Heidelberg. <https://doi.org/10.1007/978-3-642-05076-3>
- Gavlak, R., Horneck, D., & Miller, R. O. (2005). *Soil, plant and water reference methods for the western region* (3rd ed.). Western Coordinating Committee.
- Gee, G. W., & Bauder, J. W. (1986). Particle-size analysis. In A. Klute (Ed.), *Methods of soil analysis, part 1. Physical and mineralogical methods* (pp. 383-411). Soil Science Society of America, American Society of Agronomy. <https://doi.org/10.2136/sssabookser5.1.2ed.c15>
- Gupta, H. V., Kling, H., Yilmaz, K. K., & Martinez, G. F. (2009). Decomposition of the mean squared error and NSE performance criteria: Implications for improving hydrological modelling. *Journal of Hydrology*, 377(1-2), 80-91. <https://doi.org/10.1016/j.jhydrol.2009.08.003>
- Heiniger, R., McBride, R., & Clay, D. (2003). Using soil electrical conductivity to improve nutrient management. *Agronomy Journal*, 95(3), 508-519. <https://doi.org/10.2134/agronj2003.0508>
- Herndon, E., Kinsman-Costello, L., Domenico, N. D., Duroe, K., Barczok, M., Smith, C., & Wullschleger, S. D. (2020). Iron and iron-bound phosphate accumulate in surface soils of ice-wedge polygons in arctic tundra. *Environmental Science: Processes & Impacts*, 22(7), 1475-1490. <https://doi.org/10.1039/D0EM00142B>
- Hossain, Md. D., Kashem, M. A., & Mustary, S. (2023). IoT based smart soil fertiliser monitoring and ML based crop recommendation system. *2023 International Conference on Electrical, Computer and Communication Engineering (ECCE)*, 1-6. <https://doi.org/10.1109/ECCE57851.2023.10100744>

- Islam, M. R., Oliullah, K., Kabir, M. M., Alom, M., & Mridha, M. F. (2023). Machine learning enabled IoT system for soil nutrients monitoring and crop recommendation. *Journal of Agriculture and Food Research*, 14, Article 100880. <https://doi.org/10.1016/j.jafr.2023.100880>
- Klute, A. (1986). Water Retention: Laboratory Methods. In A. Klute (Ed.), *Methods of soil analysis, part 1. Physical and mineralogical methods* (pp. 635-662). Soil Science Society of America, American Society of Agronomy. <https://doi.org/10.2136/sssabookser5.1.2ed.c26>
- Mazur, P., Gozdowski, D., & Wnuk, A. (2022). Relationships between soil electrical conductivity and sentinel-2-derived NDVI with pH and content of selected nutrients. *Agronomy*, 12(2), Article 354. <https://doi.org/10.3390/agronomy12020354>
- Miyamoto, T., Kameyama, K., & Iwata, Y. (2015). Monitoring electrical conductivity and nitrate concentrations in an Andisol field using time domain reflectometry. *Japan Agricultural Research Quarterly: JARQ*, 49(3), 261-267. <https://doi.org/10.6090/jarq.49.261>
- Nadporozhskaya, M., Kovsh, N., Paolesse, R., & Lvova, L. (2022). Recent advances in chemical sensors for soil analysis: A review. *Chemosensors*, 10(1), Article 35. <https://doi.org/10.3390/chemosensors10010035>
- Nottingham, A. T., Gloor, E., Bååth, E., & Meir, P. (2022). Soil carbon and microbes in the warming tropics. *Functional Ecology*, 36(6), 1338-1354. <https://doi.org/10.1111/1365-2435.14050>
- Nyakuri, J. P., Bizimana, J., Bigirabagabo, A., Kalisa, J. B., Gafirita, J., Munyaneza, M. A., & Nzemerimana, J. P. (2022). IoT and AI based smart soil quality assessment for data-driven irrigation and fertilisation. *American Journal of Computing and Engineering*, 5(2), 1-14. <https://doi.org/10.47672/ajce.1232>
- Obalum, S. E., Watanabe, Y., Igwe, C. A., Obi, M. E., & Wakatsuki, T. (2013). Improving on the prediction of cation exchange capacity for highly weathered and structurally contrasting tropical soils from their fine-earth fractions. *Communications in Soil Science and Plant Analysis*, 44(12), 1831-1848. <https://doi.org/10.1080/00103624.2013.790401>
- Omonode, R., & Vyn, T. (2006). Spatial dependence and relationships of electrical conductivity to soil organic matter, phosphorus, and potassium. *Soil Science*, 171(3), 223-238. <https://doi.org/10.1097/01.ss.0000199698.94203.a4>
- Othaman, N. N. C., Isa, M. N., & Hussin, R. (2021). IoT based soil nutrient sensing system for agriculture application. *International Journal of Nanoelectronics and Materials*, 14, 279-288.
- Pansu, M., Gautheyrou, J., Pansu, M., & Pansu, M. (2006). *Handbook of soil analysis: Mineralogical, organic and inorganic methods*. Springer. <https://doi.org/10.1007/978-3-540-31211-6>
- Reddy, Mr. Ch. S., Dasari, A., Meghna, G., Lekhna, S., & Shriya, S. (2023). Soil fertility analysis using IoT. *International Journal for Research in Applied Science and Engineering Technology*, 11(6), 3738-3743. <https://doi.org/10.22214/ijras.2023.54183>
- Reeuwijk, L. P. van. (2002). *Procedures for soil analysis* (6th ed.). International Soil Reference and Information Centre.
- Rhoades, J. D., Manteghi, N. A., Shouse, P. J., & Alves, W. J. (1989). Soil electrical conductivity and soil salinity: New formulations and calibrations. *Soil Science Society of America Journal*, 53(2), 433-439. <https://doi.org/10.2136/sssaj1989.03615995005300020020x>

- Rout, P. P., & Arulmozhiselvan, K. (2019). Effect of soil texture on drying pattern of soil moisture after saturation. *International Journal of Current Microbiology and Applied Sciences*, 8(3), 697-704. <https://doi.org/10.20546/ijcmas.2019.803.086>
- Sahu, G., Das, S., & Mohanty, S. (2020). Nutrient budgeting of primary nutrients and their use efficiency in India. *International Research Journal of Pure and Applied Chemistry*, 21(11), 92-114. <https://doi.org/10.9734/irjpac/2020/v21i1130227>
- Schulten, H.-R., & Schnitzer, M. (1997). The chemistry of soil organic nitrogen: A review. *Biology and Fertility of Soils*, 26(1), 1-15. <https://doi.org/10.1007/s003740050335>
- Seyedmohammadi, J., Navidi, M. N., & Esmaeelnejad, L. (2019). Geospatial modeling of surface soil texture of agricultural land using fuzzy logic, geostatistics and GIS techniques. *Communications in Soil Science and Plant Analysis*, 50(12), 1452-1464. <https://doi.org/10.1080/00103624.2019.1626870>
- Tamad, T., Ismangil, I., & Maryanto, J. (2020). The biochemical characteristics of phosphate bacteria capable of increasing soil phosphorus bioavailability in Andisols. *Soil Science Annual*, 71(2), 125-132. <https://doi.org/10.37501/soilsa/122403>
- Thiemig, V., Rojas, R., Zambrano-Bigiarini, M., & De Roo, A. (2013). Hydrological evaluation of satellite-based rainfall estimates over the Volta and Baro-Akobo Basin. *Journal of Hydrology*, 499, 324-338. <https://doi.org/10.1016/j.jhydrol.2013.07.012>
- Yu, A. J., Lin, X., Zhu, J., He, H., & Li, L. (2023). Environmental effects on ammonium adsorption onto clay minerals: Experimental constraints and applications. *Applied Clay Science*, 246, Article 107165. <https://doi.org/10.1016/j.clay.2023.107165>
- Zhao, X., Wang, J., Zhao, D., Sefton, M., & Triantafyllis, J. (2022). Mapping cation exchange capacity (CEC) across sugarcane fields with different comparisons by using DUALEM data. *Journal of Environmental and Engineering Geophysics*, 27(4), 191-205. <https://doi.org/10.32389/JEEG22-002>
- Žinete, J. (2023). The role of NPK fertilisers in modern agriculture: Fuelling crop growth and maximising yields. *International Journal of Manure and Fertiliser*, 11(2).

Optimised Ultrasound-Assisted Alkaline Extraction and Rheology of Chia Mucilage with Co-Solutes

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ABSTRACT

Chia mucilage is a promising food additive with applications in food systems. However, its attachment to seeds hinders the extraction and utilisation of the mucilage. Therefore, the study aims to optimise chia mucilage extraction using ultrasound and alkaline treatment to enhance yield, hue angle, and apparent viscosity and evaluate its rheological behaviour in the presence of co-solutes (sucrose and sodium chloride). Chia seeds were dispersed in distilled water, and the suspension was modulated to various pH values (7-9) in an ultrasound bath at varying temperatures (50-70 °C) and duration (30-60 minutes). Response Surface Methodology identified the optimum conditions (67.2 °C, 32.7 minutes, pH 9), yielding 79.62% with a hue angle of 74.79° and viscosity of 0.82 Pa.s. The optimised (M2) chia mucilage solution (CMS) was validated and showed higher yield

and hue angle than the macerated (M1) samples. Rheological evaluation with incorporation of sucrose (0-20%) and NaCl (0-250mM) showed non-Newtonian pseudoplastic behaviour, with 20% sucrose exhibiting the lowest k value in M1, yet the highest in M2. The relatively stable consistency coefficient value in M2, despite the addition of sucrose from 1 to 20%, indicates the suitability of ultrasonication and alkaline pH during the extraction process. Moreover, the addition of salt exerted a significant negative effect on the apparent viscosity of M1 samples while exerting the opposite effect on M2 CMS. These findings demonstrate that ultrasound-

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assisted alkaline extraction improves both yield and functional stability of chia mucilage, and its rheological properties were strongly influenced by sucrose and salt concentrations, indicating strong potential as a functional food hydrocolloid for diverse food formulation applications.

Keywords: Alkaline extraction, chia mucilage, co-solutes, optimisation, sonication, sonication time, sonication temperature

INTRODUCTION

The food hydrocolloid market is expected to grow significantly, focusing on sustainability, health benefits, and sensory perceptions (Saqib et al., 2022). Natural mucilages, also known as hydrocolloids, are more appealing than synthetic thickeners, such as xanthan and guar gum, owing to their cost-effectiveness, advanced biocompatibility, and non-toxicity (de Campo et al., 2017; Timilsena et al., 2015). According to Coorey et al. (2014) water and oil retention capacities and emulsion stability of chia mucilage (CM) were higher compared to commercially used guar gum. This makes chia mucilage an excellent foam stabiliser, emulsifier or thickener in the food industry (Coorey et al., 2014; Muñoz et al., 2012).

Previously, conventional extraction of chia mucilage was optimised in ranges; extraction temperature (70-85 °C), water: seed ratio (14-40:1), pH 8 and extraction time (2-2.4 h) using centrifugation, vacuum filtration, as well as drying processes (Campos et al., 2016; Chavan et al., 2019; Muñoz et al., 2012). Ultrasonication, a promising environmentally friendly treatment, has proven to be efficient for the extraction and modification of hydrocolloids, attributing to its high reproducibility, lower extraction time for highly efficient extractions and effective extraction of tightly bound hydrocolloids (Silva et al., 2022). According to Maran and Priya (2014), ultrasound creates cavitation bubbles that facilitate breaking of the matrix of the seed connecting to the desired product, hence encouraging mass transfer from matrix to solvent, which yields superior quality of material in less time.

The extraction yield, physicochemical and also functional properties of hydrocolloids, including chia, *Hypytis suaveolens* (L.) Poit, and *Salvia macrosiphon* can be highly affected by sonication conditions such as time, temperature, pH, seed: water ratio and sonication power (Farahnaky et al., 2013; Morales-Tovar et al., 2020; Silva et al., 2022). The optimisation of sonication conditions (Morales-Tovar et al., 2020; Silva et al., 2022) are necessary to make the ultrasound-assisted extraction (UAE) procedure more efficient and cost-effective (Hedayati et al., 2021), as well as to produce a greater yield of the desired product without compromising the necessary properties of hydrocolloids.

For industrial applications, real food formulations typically require the incorporation of additional ingredients, for instance, sugar and salt. The functionalities of food polymers within food systems are influenced by their interactions with sugars, salts and proteins

(Dickinson, 2003). Various studies have examined the impact of hydrocolloids, including quince seed, sage seed, balangu seed, qodume shahri seeds and *Hyptis suaveolens* L. seed and its interactions with salts and sugars (Farahmand et al., 2021; Koocheki et al., 2013; Pérez-Orozco et al., 2019; Yousefi et al., 2014).

Despite the promising functional properties of chia mucilage, its strong adherence to the seed coat limits efficient extraction using conventional methods, which are often time-consuming and may compromise quality. To the best of our knowledge, few studies have utilised UAE in the extraction of chia mucilage (Saporittis et al., 2023; Silva et al., 2022; Urbizo-Reyes et al., 2019), as current extraction approaches do not adequately address the optimisation of process conditions under combined ultrasound and alkaline environments, especially in a solution form without any drying step conducted. With the integration of ultrasound-assisted extraction with alkaline pH conditions, the yield and functional properties of chia mucilage could be enhanced while maintaining its structural integrity.

In addition, the behaviour of chia mucilage in the presence of common food co-solutes such as sugars and salts remains largely unexplored. Furthermore, there is a strong possibility that the optimised mucilage will exhibit stable and desirable rheological behaviour in the presence of co-solutes, which is critical for predicting its performance in real food systems. Determining this behaviour is significant in ensuring consistency, functionality, and adaptability of chia mucilage across varying formulation conditions. A key challenge lies in balancing process intensification with the preservation of its functional characteristics. Thus, this study aimed to optimise the integrated ultrasound-assisted alkaline extraction (UAE) of chia mucilage solution (CMS) with response surface optimisation without drying and evaluate the impact of the addition of sugar and salt on the rheological characteristics of the solution. By gaining insights into these factors, we can better utilise chia mucilage as a sustainable and functional ingredient in various food formulations.

MATERIALS AND METHOD

Chia Seed Collection and Preparation

Black chia seeds (*Salvia hispanica* L.) were provided by The Chia Company (Victoria, Australia). Distilled water was used for all solution preparations. Sodium hydroxide (analytical grade) was obtained from R&M Chemicals (Malaysia). Sucrose and sodium chloride (analytical grade) were purchased from Chemiz (Malaysia).

Chia Mucilage Solution (CMS) Extraction

Chia seeds were hydrated in distilled water at a ratio of 1:40 (seed-to-water) with constant stirring for 2 h (Orifici et al., 2018). Following that, the dispersion was subjected to ultrasound and pH treatment as described below. After treatment, the samples were vacuum filtrated using cheesecloth and a chia mucilage solution (CMS) was obtained.

Conventionally extracted CMS was prepared as a control with a similar seed-to-water ratio and stirring time (maceration only) to evaluate the differences between the optimised and conventionally extracted samples.

The gel solutions were kept at 4 °C till further analysis. According to the preliminary studies, the yield of the mucilage solution was greater at this seed-to-water ratio (1:40) compared to other seed-to-water ratios (1:10-50) and the hydration period was fixed at this limit as chia mucilage gel reaches its full potential after 2 hours of immersion according to Muñoz et al. (2012). pH values were fixed based on the preliminary studies, as well as the observations from Capitani et al. (2015) and Muñoz et al. (2012) in which, at higher pH values (8-9), higher chia mucilage yield values were observed due to the increased solubility of chia mucilage at alkaline conditions, allowing the mucilage to be extracted easily.

Optimisation of CMS using Ultrasound and Alkaline Treatment

To fix the optimal condition of ultrasound and alkaline parameters, the impact of three independent variables including pH (X_1 : 7, 8, 9), sonication time (X_2 : 20, 45, 60 minutes) and sonication temperature (X_3 : 50, 60, 70 °C) on the dependent variables (Y), extraction yield (%), hue angle (°) and apparent viscosity (Pa.s) were analysed through RSM using Minitab software. The hydrated chia mucilage samples were treated with ultrasonication using an ultrasonic bath (Elmasonic S100H, Germany) operating at a frequency of 37 kHz and with a recirculation bath.

Experimental design included a three-level, three-factor design, comprising a randomised 15 experimental runs, in which the centre point was repeated three times.

For each response, ANOVA was performed to identify the significant model terms. The experimental design matrix by the BBD and the corresponding experiment values are tabulated in Table 1.

The response optimiser plot in Minitab was utilised to establish the optimal factor levels, with targets defined for all variables and responses. Under recommended optimum conditions, the adequacy of the model was verified by conducting experiments (Bazezew et al., 2022).

Yield Analysis

Yield percentage (%) of CMS was calculated as the ratio of the final mass after vacuum filtration to the initial mass after dispersion, multiplied by 100.

Colour

Colour parameters were identified using a colourimeter (Konica Minolta, Model No. CR-410, Japan). Measurements were obtained in the $L^*a^*b^*$ scale, and the hue angle value was calculated (Campos et al., 2016).

Table 1
Box Behnken Design (BBD) with experimental values for the extraction of mucilage

Sample	pH (A)	Sonication Time (min, B)	Sonication Temperature (°C, C)	Yield (%)	Hue Angle (°)	Apparent Viscosity, η (Pa.s)
1	7	60	60	78.7417	74.4606	0.4205
2	8	30	50	80.7917	45.0000	0.2379
3	7	45	70	82.6750	70.2239	0.1485
4	8	60	50	75.7500	64.5821	0.3966
5 _c	8	45	60	80.3250	64.9439	0.4395
6 _c	8	45	60	78.4000	52.6333	0.4389
7	8	60	70	77.0000	72.1157	0.1493
8	9	60	60	75.5500	73.6476	0.5067
9	7	45	50	77.5500	64.3793	0.5727
10	9	30	60	80.0417	65.0809	0.5034
11	8	30	70	82.2250	72.8476	0.0849
12	9	45	70	77.9250	75.5878	0.7658
13	9	45	50	83.7000	54.2356	0.6689
14 _c	8	45	60	76.4750	63.4349	0.5412
15	7	30	60	84.1250	59.1986	0.2641

Note. c = Centre point

Rheology

The rheological measurements were carried out in a dynamic controlled stress rheometer (Haake RS600, ThermoElectron, Germany). For the apparent viscosity, the PP35 Ti plate was used with measurements executed under a shear rate range of 0 to 300 s⁻¹ within 3 minutes at 25 ± 1 °C. For the addition of co-solutes, a PP60 Ti plate was used. CMS solutions were allowed to equilibrise at 25 °C for 3 minutes at 3 s⁻¹ and then followed by a linear increasing shear rate from 1 to 100 s⁻¹ in 3 minutes. As suggested by previous studies, the power law model was used to obtain the measurements (Capitani et al., 2015; Timilsena et al., 2015).

Addition of Co-Solutes in CMS

Addition of co-solutes such as sucrose and sodium chloride was evaluated in CMS extracted using two different methods, macerated (M1) and optimised (M2) CMS. The macerated CMS was previously used as a control to evaluate the difference between the conventionally extracted and the optimised CMS. To reiterate, M1 samples were not subjected to any ultrasound or pH treatment while M2 samples underwent the optimised treatment determined at pH 9, 32.7 minutes at 67.2 °C based on high yield, hue angle and viscosity.

Different concentrations of sucrose (0, 1, 5 and 20%) and NaCl (0, 10, 50 and 250mM) were added to the freshly extracted mucilage solutions, under constant magnetic stirring for 10 min. A total of two controls, one for each method (M1 and M2) without any addition of co-solutes (0% or 0mM), were prepared. The respective control samples were used as the baseline for evaluating the effects of the various sucrose and NaCl concentrations on the mucilage solutions extracted using different methods. The concentration of sucrose and salt was selected according to Yang et al. (2020) and Yousefi et al. (2014)'s studies, respectively.

Statistical Analysis

The statistical analyses were executed using Minitab 19. A two-way ANOVA with Tukey's test was carried out to distinguish the effect of method type and varying concentrations of sucrose and salt. Modelling of rheological data was performed using Microsoft Excel 16 (Solver).

RESULT AND DISCUSSION

Optimisation of US-Assisted Alkaline Mucilage Solution Extraction Parameters and Model Fitting for Response Surface

Table 2 displays the linear, quadratic, interaction terms and statistical parameters (R^2 , Adj- R^2 , p- and f-values) after removing the effect of non-significant factors for the process variables. The ANOVA analysis shows that lack of fit was not significant ($p > 0.05$) and the high R^2 values demonstrate the model's suitability in predicting the responses (yield, hue angle and apparent viscosity).

Effect of Independent Variables on Mucilage Yield

After removing the non-significant terms, the linear effect of sonication time and the interaction effects of pH and sonication temperature had significant effects ($p < 0.05$) on the extraction yield (Table 2).

The regression equation of mucilage yield (Y_1) is as in Equation 1:

$$Y_1 = 75.2 - 6.9X_1 - 0.207X_2 + 1.39X_3 + 1.368X_1^2 - 0.00068X_2^2 + 0.006956X_3^2 + 0.0149X_{12} - 0.2725X_{13} - 0.00031X_{23} \quad [1]$$

Table 2
Analysis of variance (ANOVA) for fitting the model of yield, hue angle, and apparent viscosity

Source	Response Variables		
	Yield (% <i>, Y₁</i>)	Hue Angle (° <i>, Y₂</i>)	Apparent Viscosity, η (Pa.s, <i>Y₃</i>)
Linear			
x ₁	0.201	0.981	0.004*
x ₂	0.001*	0.009*	0.124
x ₃	0.644	0.001*	0.017*
Quadratic			
x ₁₁	0.122	0.055	0.016*
x ₂₂	-	0.278	0.005*
x ₃₃	-	-	0.125
Interaction			
x ₁ x ₂	-	-	0.345
x ₁ x ₃	0.006*	0.111	0.016*
x ₂ x ₃	-	0.048*	0.549
R ²	0.82	0.89	0.95
Adj-R ²	0.72	0.77	0.86
p-value	0.002*	0.007*	0.009*
f-value	9.83	7.86	10.81
Lack-of-fit (p-value)	0.625	0.956	0.361

Note. *Significant at $p < 0.05$. x₁ to x₃ denote pH, sonication time, and temperature, respectively; x₁², x₃² and x₁x₂, x₁x₃, x₂x₃ represent quadratic and interaction terms, respectively

Contour plots generated by the model in Figure 1 (a-c) represent the correlation between independent variables and yield. The combined effect of pH and sonication time affected the chia yield, as depicted in Figure 1a. A decrease in yield can be seen when sonication time increased from 30 to 60 minutes at pH 7 and 9. Similarly, an increase in pH to 9 led to a declining yield at all time ranges. The highest value of pH (9) and sonication time (60 minutes) at 60 °C resulted in the lowest yield (75.55%). Meanwhile, the lowest pH (7) and sonication time (30 mins) at 60 °C yielded the highest at 84.13%. Higher yield value at pH 7 could be from hydrolysis, in which certain insoluble polysaccharide fractions could have been converted to soluble ones (Karazhiyan et al., 2011).

Maran and Priya (2014) explained that the cavitation effects of the ultrasonic waves accelerate the swelling as well as the hydration. Hence, the number of cells exposed to the extraction medium (aqueous) increases and causes disruption. Hence, polysaccharides are easily released into the medium, and the yield is enhanced in a shorter time. A longer sonicating time leads to overexposure to ultrasound treatment, causing structural and polysaccharide ruin. Moreover, this leads to the loss of soluble mucilage components

during the extraction process, resulting in lower overall yields compared to shorter sonication times (Urbizo-Reyes et al., 2019). Therefore, increasing the sonication time is both time-consuming and an unreasonable situation from an economic point of view (Şahin & Şamlı, 2013).

Likewise, as seen in Figure 1b, at pH 7, the yield greatly rose from 77.55 to 82.68% at rising temperatures, while giving an opposite result at pH 9. As the interaction between pH and sonication temperature has a significantly ($p < 0.05$) negative effect (Table 2) on yield, it can be deduced that a higher yield can be obtained at a higher temperature at pH 7 and a lower temperature at pH 9. As chia mucilage contains a huge quantity of uronic acids, a lower extraction rate can be obtained under acidic conditions (Balke & Diosady, 2000). Hence, at alkaline pH, a higher yield is observed.

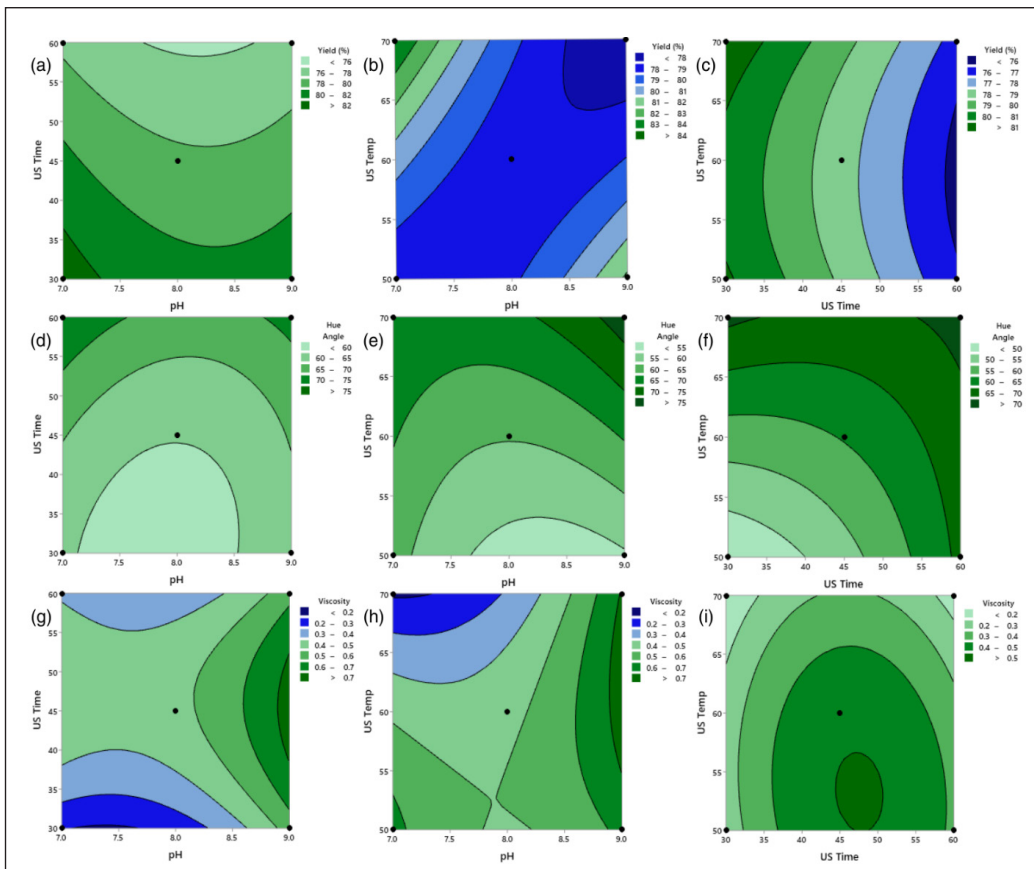


Figure 1. Contour plots of extraction yield (a-c), hue angle (d-f) as well as viscosity (g-i) explaining the relationship of pH and ultrasonication time (a, d, g - sonication temperature = 60 °C), pH and sonication temperature (b, e, h - sonication time = 45 minutes) and sonication time and temperature (c, f, i - pH = 8)

A decline is noticed as sonication time increases at all temperatures, i.e., 50 to 70 °C (Figure 1c). Conversely, as the temperature rises from 50 to 70 °C, a slight increase in yield can be seen both at 30 and 60 minutes. This correlation strongly indicates the favourable effect of sonication temperature on mucilage yield. The collective effect of temperature and time can be explained by the mass transfer effect, in which the mucilage diffuses at a higher rate. The effect of time will be more pronounced at higher temperatures (50-65 °C), so a higher extraction time can cause detrimental effects in the polysaccharides' structure and decrease the yield.

Effect of Independent Variables on Hue Angle

Linear effect of sonication time and temperature, as well as the interactive effects between pH and sonication time, had significant effects ($p < 0.05$) on hue angle (Table 2). Koocheki et al. (2009) suggested that the colour of the mucilage may be attributed to the leaching of natural pigments or tannic substances from the coat of chia.

The regression equation for hue angle (Y_2) is given as in Equation 2:

$$Y_2 = 426 - 100.0X_1 + 2.22X_2 - 1.58X_3 + 5.11X_1^2 + 0.0118X_2^2 + 0.0065X_3^2 - 0.112X_{12} + 0.388X_{13} - 0.0339X_{23} \quad [2]$$

As shown in Figure 1d, while the hue angle considerably rose at 30 minutes, only a slight decline was noticed at 60 minutes. Conversely, a significant rise in hue angle was observed as sonication time increased from 30 to 60 minutes, both at pH 7 and 9. At pH 8, when sonication time was held steady at 45 minutes, the hue angle ranged from 52.63 to 64.94°. Koocheki et al. (2009) found that at acidic conditions and a higher water-to-seed ratio, the colour of the *Lepidium perfoliatum* gum was almost yellow. This explains that the pigments were highly soluble in alkaline conditions or dissolved at lower pHs. The gradated pigment in a lower water-to-seed ratio reached saturation, and thus the hue angle did not change with pH.

Meanwhile, a linear increase in hue angle was observed when sonication temperature increased at both pH 7 and 9 (Figure 1e). The highest hue angle (75.59°) was obtained at the highest pH (9) and sonication temperature (70 °C). The hue angle obtained in this study (65.51-71.46°) is lower than that of chia mucilage powder (84.03°) but higher than that of *Lepidium perfoliatum* powder (60.76°), indicating that this mucilage possesses a yellow colour (Campos et al., 2016; Koocheki, Taherian, et al., 2009). Studies conducted on white mustard seed mucilage (Balke & Diosady, 2000) and *P. flexuosa* seed gum (Ibañez & Ferrero, 2003) concluded that a highly coloured product was obtained at a higher temperature and alkaline medium, respectively.

Effect of Independent Variables on Apparent Viscosity

As seen from Table 1, all the samples display $n < 1$, implying the characteristics of a shear-thinning liquid of a non-Newtonian behaviour and the values of apparent viscosity, η , were obtained spanning between 0.08 and 0.77 Pa.s. The apparent viscosity was significantly ($p < 0.05$) affected by the linear effects of pH and sonication temperature, quadratic effects of pH and time, as well as the interaction between pH and temperature, as referred in Table 2.

The regression equation for apparent viscosity (Y_3) is as in Equation 3:

$$Y_3 = 9.26 - 2.715X_1 + 0.1073X_2 - 0.0218X_3 + 0.1361X_1^2 - 0.000825X_2^2 - 0.000704X_3^2 - 0.00255X_{12} + 0.01303X_{13} - 0.000157X_{23} \quad [3]$$

Upon an increment in pH values, an increase in viscosity was observed at both 30 mins (0.26 to 0.50 Pa.s) and 60 minutes (0.42 to 0.51 Pa.s) (Figure 1g). The graphs also show that at pH 7 and 9, the viscosity showed a notable increase when the sonication time rose from 30 to 45 minutes. However, when the sonication time reached 60 minutes, the viscosity showed a slight decrease. It can be deduced that apparent viscosity peaks at 45 minutes and decreases when sonicated for a longer period of time.

The quadratic effect between pH and sonication temperature significantly ($p < 0.05$) affected the mucilage viscosity as depicted in Figure 1h. Though viscosity values increased at both 50 and 70 °C as the pH increased, a substantial rise was noted, particularly at 70 °C, the values spanning from 0.15 to 0.77 Pa.s. For temperature variations, at pH 7, a temperature rise led to a decrease in viscosity values, while the opposite happened at pH 9. Notably, the highest viscosity (0.77 Pa.s) is obtained when both pH and temperature are at the highest range, at 9 and 70 °C.

A negative curvilinear trend was observed at increasing sonication time of mucilage extraction (Figure 1i). When the sonication temperature was set at the highest temperature (70 °C), and the shortest time (30 minutes) at pH 8, the viscosity was lowest at 0.08 Pa.s. Generally, when the sonication temperature reaches to 70 °C, parameters such as viscosity as well as surface tension of water (solvent) decrease, thereby decreasing the sonication effect (Zia et al., 2022). The above results suggest that the mucilage fibres were progressively separated from the seed and freed to the aqueous solution, hence the high yield and lower viscosity. This can be seen in run 11, which has a relatively high yield (82.23%) but contributed to the lowest viscosity (0.08 Pa.s). The study also suggests that a sonication temperature up to 65 °C gives a higher viscosity value, and further increase may cause a decline in the viscosity value.

Silva et al. (2022) discovered that a rise in stirring or sonication time led to a decrease in apparent viscosity and k value. 30 min sonication caused a drop in viscosity. This

statement is slightly contradictory to this study, as viscosity increased when the mucilage was sonicated for 45 minutes. This could be due to the differences in ultrasound type (probe and bath), as the ultrasound probe could allow for a more precise and localised delivery of ultrasound energy as opposed to the ultrasound bath, hence giving a more intense cavitation effect, leading to faster breakdown of molecular structures and reduction in viscosity (Chemat et al., 2011).

Optimisation and Model Verification

In this study, 15 formulations were optimised by BBD based on a composite desirability (D) value of 0.7135. This optimisation study was planned for maximum extraction yield, hue angle and apparent viscosity in ultrasonicated alkaline chia mucilage. The predicted most desirable set of ultrasound extraction conditions was pH (9), sonication time (32.7 min) and temperature (67.2 °C) as presented in Table 3. Based on the suggested extraction conditions, the predicted favourable values attained were maximum extraction yield of 79.42%, maximum hue angle of 75.97° and maximum viscosity of 0.61 Pa.s (Table 4). Experimental validation was performed to confirm the adequacy and accuracy of the most desirable set of extraction conditions. When comparing the experimental to predicted results, a similar yield value was obtained, with slightly lower hue angle and higher apparent viscosity. Though one sample t-test indicated no significant differences (p>0.05) observed in all the responses to the predicted value. This has proven that pH 9, sonication at 32.7 minutes and 67.2 °C is the most desirable set of conditions for ultrasonic extraction of mucilage solution.

Table 3
Predicted optimal condition for CMS extraction

Variables	Minimum	Maximum	Optimum
pH	7	9	9
Sonication Time (min)	30	60	32.7
Sonication Temperature (°C)	50	70	67.2

Table 4
Predicted and experimental values for the optimum extraction parameters

Responses	Predicted Value	Experimental Values ^a	p-value
Yield (%)	79.4206	79.616 ± 0.421	0.507*
Hue Angle	75.9703	74.791 ± 0.611	0.079*
Apparent Viscosity (Pa.s)	0.6129	0.8182 ± 0.1072	0.08*

Note. ^aMean (n = 3), *Not significant at p < 0.05

Comparison Between Optimised and Control Extracted Mucilage Solution

The optimised mucilage solution sonicated at pH 9 for 32.7 minutes at 67.2 °C was compared with a macerated control sample in terms of extraction yield, colour parameters (hue angle, L^* , a^* , b^*) and rheological parameters such as η (apparent viscosity), k and n values (Table 5). This provides a reference on the impact of sonication on the mucilage solution compared to a non-sonicated sample.

The extraction yield of the optimised mucilage solution is significantly higher at 79.62% compared to the control, 75.89%. Several studies agree that ultrasound offers a greater yield than the conventional method, as seen in sage and chan seeds (Farahnaky et al., 2013; Morales-Tovar et al., 2020). Moreover, the optimised yield obtained in this study (79.62%) is much higher than the wet yield reported by Chaves et al. (2018) after centrifugation (18.25%). From centrifugation, only the gel is obtained, excluding the liquid and seeds, whereas vacuum filtration filters out the seeds only, leaving the mucilage in a liquid form. Hence, the higher yield in this study.

Comparing the colour parameters of the samples, the hue angle of the optimised sample is significantly higher, while a^* and b^* values were significantly lower. Moreover, no significant differences were observed in any of the rheological parameters between the control and the optimised sample. This is the opposite of Farahnaky et al. (2013) in which the apparent viscosity of the sage mucilage from the conventional extraction method was greater than that of the sonicating probe. To reiterate, the cavitation effect of the ultrasonic waves increases the yield of mucilage solution as well as elevates the dissemination of natural pigments from chia, leading to a higher hue angle, and the viscosity increases at alkaline conditions. Hence, ultrasound can be employed to facilitate the easy extraction of chia mucilage tightly bound to the seed, leading to functional, textural, and economic benefits. It allows for the incorporation of mucilage into an array of food products, aiding in their quality, appeal, and overall marketability.

Table 5
Experimental values for control and optimised mucilage

Analysis	Control	Optimised
Yield (%)	75.892 ^b ± 0.667	79.616 ^a ± 0.421
Hue Angle (°)	72.749 ^b ± 0.598	74.791 ^a ± 0.611
L^*	70.217 ^a ± 0.015	69.377 ^a ± 1.563
a^*	1.8033 ^a ± 0.025	1.2767 ^b ± 0.015
b^*	4.700 ^a ± 0.021	0.3833 ^b ± 0.161
Apparent Viscosity, η (Pa.s)	0.7863 ^a ± 0.066	0.8183 ^a ± 0.107
k (Pa.s ⁿ)	0.0502 ^a ± 0.007	0.0489 ^a ± 0.004
n	0.5211 ^a ± 0.009	0.5211 ^a ± 0.010

Note. ^a Means in the same row with the same letters are not significantly different at $p < 0.05$ ($n = 3$)

Effect of Sucrose

Referring to Table 6, the Power Law parameters such as the flow behaviour index (*n*), consistency coefficient (*k*), apparent viscosity (η) and R^2 values.

The high R^2 (0.95-0.97) denotes a good fit for the Power Law model (Marcotte et al., 2001). CMS’s rheological behaviour was characterised based on the addition of sucrose, as well as how the differences in the method of extraction affect its fluid behaviour.

Table 6 shows that the concentration of sucrose and the interaction between the extraction method and the sucrose concentration had significant effects on *k*, *n* and η values. The *n* values displayed a value of less than 1, hence the samples exhibit shear-thinning behaviour. A significant difference between the *n* value according to the method of extraction was observed only at 0%. The *n* value of M2 was significantly ($p<0.05$) higher than that of M1 at 0%, indicating that the sonicated CMS had a lower shear-thinning effect.

Table 6
Effect of type of extraction (*T*), sucrose concentration (*C*) and their interactions (*T* × *C*) on Power Law parameters for chia mucilage solution

Analysis	C (%)	T		p-value		
		M1	M2	T	C	T × C
<i>k</i> (Pa.s ⁿ)	0	0.104 ^{aA}	0.095 ^{abA}	ns	**	**
	1	0.075 ^{abB}	0.106 ^{aA}			
	5	0.103 ^{aA}	0.078 ^{bB}			
	20	0.043 ^{bB}	0.076 ^{bA}			
<i>n</i>	0	0.461 ^{bB}	0.505 ^{bcA}	ns	**	*
	1	0.505 ^{abA}	0.492 ^{cA}			
	5	0.517 ^{abA}	0.516 ^{bA}			
	20	0.577 ^{aA}	0.554 ^{aA}			
η (Pa.s)	0	1.52 ^{aA}	1.048 ^{bB}	ns	**	**
	1	0.935 ^{bA}	1.219 ^{aA}			
	5	1.140 ^{abA}	0.875 ^{cA}			
	20	0.393 ^{cB}	0.640 ^{dA}			
R^2	0	0.970	0.961			
	1	0.963	0.959			
	5	0.972	0.953			
	20	0.962	0.965			

Note. *Lowercase letters within a column denote no significant differences among sucrose-added samples. Uppercase letters within rows denote no significant difference between samples from different extraction methods at each concentration of sucrose. The control sample was prepared at 0%. (Mean = 2). ¹ANOVA: ** $p<0.01$; * $p<0.05$; ns, not significant. T × C, the interaction between the type of extraction and the concentration of sucrose

A general increasing trend in n value was observed in both M1 and M2 upon increasing sucrose concentration. At 20% sucrose concentration, a pronounced ($p < 0.05$) n value was observed for both M1 and M2 (0.577 and 0.554, respectively). Hence, at increasing sucrose concentrations, the n value increased regardless of the extraction method, indicating a reduction in shear thinning behaviour of mucilage at these concentrations.

From this study, a greater flow behaviour index (n) was obtained at increasing sucrose addition, indicating the solutions were less pseudoplastic. The addition of sucrose leading to a less pseudoplastic behaviour (higher n value) has been reported in solutions of chia, sodium alginate and *Alyssum homolocarpum* (Capitani et al., 2015; Koocheki et al., 2013; Yanes et al., 2002) and in Balangu (*Lallemantia royleana*) seed gum with glucose addition (Salehi et al., 2014). Sucrose has the ability to affect the structure and texture of gel due to its interaction with hydroxyl groups that stabilise the gel structure (Bayarri et al., 2004), hence making the gel structure more robust and less susceptible to deformation under shear stress.

Generally, a lower n value indicates higher shear-thinning behaviour (Koocheki, Mortazavi, et al., 2009) while a higher value creates a slimier mouth feeling (Szczeniak & Farkas, 1962). Hence, it is essential to use hydrocolloids possessing a low n value (Marcotte et al., 2001; Salehi et al., 2014). This is important especially in formulations of oil-in-water to inhibit drop separation but can allow the formulation to flow easily when poured (Taherian et al., 2007). Sucrose addition led to a similar increment in the n values of CMS, regardless of the extraction method.

The k values (0.043 to 0.106 Pa.sⁿ) of the sucrose-added chia mucilage solution were significantly ($p < 0.01$) affected by the sucrose concentration as well as the interaction between the type of extraction and the concentration. The consistency index (k) was found to be significantly higher in M2 at the concentrations of 1 and 20% compared to M1, though the opposite was observed at 5%. An increase in sucrose concentration led to a significant decrease in k value in both M1 and M2. This indicates that higher sucrose concentrations significantly decrease the k value, producing a less viscous and easily flowing CMS that can be used in processes such as pumping, stirring or mixing processes.

Though in M1, the addition of 20% sucrose led to the lowest k value (0.043 Pa.sⁿ) among all the formulations. Yet, at the same concentration, the k value of M2 was significantly higher (0.076 Pa.sⁿ). Moreover, the addition of sucrose, even at the highest sucrose concentration (20%), did not have a significant decline in the M2 CMS samples, as opposed to M1, which showed a significant decline. Hence, the utilisation of ultrasonication and alkaline pH during the extraction process led to samples that exhibit a relatively stable consistency coefficient value despite the addition of sucrose from 1 to 20%.

The apparent viscosity (η) of M1 samples and M2 samples was obtained from 0.393 to 1.520 Pa.s and 0.640 to 1.219 Pa.s, respectively. The addition of sucrose had a significant

effect on the η values. In both M1 and M2, the lowest viscosity was obtained at 20% sucrose level (0.393 and 0.640 Pa.s, respectively) compared to other formulations, though M2 was significantly higher than M1. The addition of sucrose at certain levels boosted the viscosity values compared to the control samples. In M1, though a significant decline in the η value was observed at increasing sugar, the addition of 5% boosted the η value comparable to the control sample. In M2, the addition of 1% sucrose in M2 significantly raised the viscosity value from 1.048 Pa.s to 1.219 Pa.s. However, further addition of sucrose caused the viscosity to decline even further. Hence, it can be concluded that the addition of sucrose significantly ($p < 0.05$) decreased the apparent viscosity of CMS. However, an increase can be expected in CMS at lower sucrose levels (5% for M1 and 1% for M2), which will then decline above the optimum levels.

The viscosity of solutions containing gums of basil seed, guar and carboxymethyl cellulose (CMC) also reduced when 15% sucrose was added (BahramParvar & Razavi, 2012). It can be inferred that the presence of sugar disrupts the network structure of the hydrocolloids, leading to a decrease in viscosity. Contrastingly, studies have shown that the apparent viscosity of polysaccharides rises at increasing sucrose concentration of *Pereskia aculeata* Miller (OPN) mucilage and balangu seed gum (Amaral et al., 2019; Salehi et al., 2014). The effect of sugar (0-50 g/100 g) concentrations on the viscosity of pectin solutions was investigated (Kar & Arslan, 1999). The authors stated that the reduced viscosity of pectin was increased by glucose and maltose up to a concentration of 30%.

The effect of sucrose-hydrocolloid interaction on the flow characteristics of real products is contingent upon the product composition, the concentration of ingredients, and the rheological attributes specific to the hydrocolloid in question (Capitani et al., 2015; Yanes et al., 2002). To conclude, the sonicated CMS samples (M2) behaved better than M1 at higher sucrose levels (20%) by exhibiting significantly higher k and η values, indicating a greater consistency and viscosity value at these concentrations.

Effect of Salt

Table 7 reports the rheological parameters of CMS that were extracted using M1 and M2, added with varying concentrations of salt. Similar to the addition of sucrose, the n values of chia mucilage solution display a value of less than 1, hence it confirms the shear-thinning behaviour of chia solution. Similar to sucrose, the extraction method had no significant effect on the rheological parameters; however, the salt concentration and the interaction between the salt levels and the extraction method yielded significance ($p < 0.05$).

Based on the comparison between the extraction methods, at lower salt concentrations (0 and 10mM), the chia mucilage samples of M2 had significantly higher n value, while the opposite was observed at higher concentrations (50 and 250mM). This explains that M1 samples presented a stronger shear thinning effect (lower n value) in lower salt

Table 7

Effect of type of extraction (*T*), salt concentration (*C*) and their interactions (*T* × *C*) on Power Law (PL) parameters for chia mucilage solution (CMS)

Analysis	C (mM)	T		T	p-value	
		M1	M2		C	T × C
<i>k</i> (Pa.s ⁿ)	0	0.104 ^{aA}	0.095 ^{aA}	ns	**	*
	10	0.072 ^{abA}	0.088 ^{abA}			
	50	0.080 ^{abA}	0.065 ^{bB}			
	250	0.047 ^{bB}	0.080 ^{abA}			
<i>n</i>	0	0.461 ^{bB}	0.505 ^{aA}	ns	**	**
	10	0.396 ^{cB}	0.484 ^{aA}			
	50	0.526 ^{aA}	0.506 ^{aB}			
	250	0.535 ^{aA}	0.409 ^{bB}			
<i>η</i> (Pa.s)	0	1.520 ^{bA}	1.048 ^{bcB}	ns	**	**
	10	2.313 ^{aA}	1.186 ^{bB}			
	50	0.821 ^{cA}	0.848 ^{cA}			
	250	0.576 ^{cB}	2.092 ^{aA}			
<i>R</i> ²	0	0.970	0.961			
	10	0.975	0.961			
	50	0.953	0.955			
	250	0.959	0.962			

Note. *Lowercase letters within a column denote no significant differences among sucrose-added samples. Uppercase letters within rows denote no significant difference between samples from different extraction methods at each concentration of sucrose. The control sample was prepared at 0%. (Mean = 2). ¹ANOVA: **p<0.01; *p<0.05; ns, not significant. T × C, the interaction between the type of extraction and the concentration of sucrose

concentrations (0 and 10mM), while M2 dominates at salt concentrations above 50mM. In M1, the addition of salt at 10mM gave a significantly lower *n* value, followed by significantly greater values at 50 and 250mM compared to the control. In contrast, no significant difference was spotted in M2 samples up to the addition of 50mM. However, the addition of 250mM significantly reduced the *n* value to 0.409. The effect of the addition of salt to *n* value behaved differently in M1 and M2 samples. The addition of salt caused a stronger shear-thinning effect in M2 samples, whereas the opposite for M1.

Assessing the influence of salt concentration on the viscosity of hydrocolloids is essential for identifying the hydrocolloid's role as a polyelectrolyte and for evaluating its functional and rheological characteristics (Koocheki, Mortazavi, et al., 2009). Electrostatic forces impact the arrangement of ionic polysaccharides and influence their physicochemical properties, including viscosity (Lin & Lai, 2009). The decreasing flow behaviour index (*n*) of M2 as the concentration of salt increases agrees with various studies (Salehi et al., 2014; Vardhanabhuti & Ikeda, 2006). Conversely, some studies found that *n* value increased

as salt concentration increased, similar to M1 extraction (Amaral et al., 2019; Koocheki, Mortazavi, et al., 2009).

Comparing the extraction methods, a significant difference in the k value was observed at higher salt concentrations, 50 and 250mM. At 50mM, M1 exhibited a higher k value than M2, and vice versa at 250mM. Besides that, a general decline in the k value can be observed at increasing salt concentration in both M1 and M2. In M1, the addition of 250mM significantly decreased the k value to 0.047, compared to M1's control sample. Meanwhile, in M2, at the same concentration (250mM - 0.080), the k value was not significantly different from its control sample (0.095). This indicates that sonicated CMS (M2) can exhibit higher viscosity despite the addition of salt at concentrations up to 250mM.

Based on the extraction methods, at lower salt concentrations (0 and 10mM), M1 yielded significantly higher viscosity (η) values than M2, and at 250mM, M2 dominated. Firstly, in M1, the addition of salt was observed to peak (significantly) at 10mM, followed by a significant decline up to 250mM compared to the control. Whereas, for M2, the addition of 50mM salt decreased the η values to 0.848 Pa.s compared to the control sample (1.048 Pa.s). However, upon adding the highest salt concentration (250mM), the viscosity shot up to 2.092 Pa.s. This concludes that the addition of salt had a significant negative effect on the apparent viscosity of conventionally extracted mucilage (M1) while exhibiting a significant positive effect on the sonicated alkaline mucilage (M2).

The reduction in viscosity in M1 at increasing salt concentration could be due to the absence of intermolecular interaction (Koocheki et al., 2009). However, at a higher concentration, multivalent ions can facilitate interaction between chains, and thus the viscosity increases. Hence, explains the increase in the viscosity of M2 at 250mM salt concentrations.

It can be concluded that macerated (M1) and ultrasonicated (M2) CMS behave differently in the presence of salts due to the different extraction methods, with the usage of ultrasonication enabling CMS to display higher viscosity at higher salt concentrations. Hence, considering the interest of utilising CMS in the food industry as an emulsion stabiliser and fat replacer, in foods containing low salt concentrations (0-50mM), M1 CMS is better to be used due to its high η , k and low n . However, for application in extreme salt concentrations (250mM), M2 behaves better as it is able to exhibit higher values and present a stronger shear-thinning effect with no significant effect on its yield stress values.

CONCLUSION

The optimisation of uS ltrasound-assisted alkaline extraction significantly improved the extraction efficiency and functional properties of chia mucilage solution. Sonication temperature, extraction time, and pH collectively influenced the yield, colour, and viscosity of the mucilage, while the optimised sample demonstrated superior yield and hue angle

compared to the conventionally extracted sample. Rheological evaluation confirmed that the mucilage exhibited non-Newtonian pseudoplastic and shear-thinning behaviour, and its properties were significantly affected by sucrose and sodium chloride concentrations. The optimised mucilage showed greater rheological stability under higher co-solute concentrations, indicating its potential application as a sustainable food hydrocolloid in complex food systems. However, this study only evaluated the effects of sucrose and sodium chloride under controlled laboratory conditions, and the functionality of the mucilage was not assessed in actual food products or during storage. Therefore, future studies should investigate the performance of optimised chia mucilage in real food formulations, as well as evaluate the influence of other co-solutes, processing conditions, and storage stability on its physicochemical and rheological properties.

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REFERENCES

- Amaral, T. N., Junqueira, L. A., Tavares, L. S., Oliveira, N. L., Prado, M. E. T., & de Resende, J. V. (2019). Effects of salts and sucrose on the rheological behaviour, thermal stability, and molecular structure of the *Pereskia aculeata* Miller mucilage. *International Journal of Biological Macromolecules*, 131, 218-229. <https://doi.org/10.1016/j.ijbiomac.2019.03.063>
- BahramParvar, M., & Razavi, S. M. A. (2012). Rheological interactions of selected hydrocolloid-sugar-milk-emulsifier systems. *International Journal of Food Science & Technology*, 47(4), 854-860. <https://doi.org/10.1111/j.1365-2621.2011.02918.x>
- Balke, D. T., & Diosady, L. L. (2000). Rapid aqueous extraction of mucilage from whole white mustard seed. *Food Research International*, 33(5), 347-356. [https://doi.org/10.1016/S0963-9969\(00\)00055-7](https://doi.org/10.1016/S0963-9969(00)00055-7)
- Bayarri, S., Durán, L., & Costell, E. (2004). Influence of sweeteners on the viscoelasticity of hydrocolloids gelled systems. *Food Hydrocolloids*, 18(4), 611-619. <https://doi.org/10.1016/j.foodhyd.2003.10.004>
- Bazezew, A. M., Emire, S. A., Sisay, M. T., & Teshome, P. G. (2022). Optimisation of mucilage extraction from *Ximenia americana* seed using response surface methodology. *Heliyon*, 8(1), Article e08781. <https://doi.org/10.1016/j.heliyon.2022.e08781>
- Campos, B. E., Dias Ruivo, T., da Silva Scapim, M. R., Madrona, G. S., & de C. Bergamasco, R. (2016). Optimisation of the mucilage extraction process from chia seeds and application in ice cream as a stabiliser and emulsifier. *LWT*, 65, 874-883. <https://doi.org/10.1016/j.lwt.2015.09.021>
- Capitani, M. I., Corzo-Rios, L. J., Chel-Guerrero, L. A., Betancur-Ancona, D. A., Nolasco, S. M., & Tomás, M. C. (2015). Rheological properties of aqueous dispersions of chia (*Salvia hispanica* L.) mucilage. *Journal of Food Engineering*, 149, 70-77. <https://doi.org/10.1016/j.jfoodeng.2014.09.043>

- Chavan, V. R., Kale, R. V., Raut, G. S., & Gadhe, K. S. (2019). Studies on extraction and characterisation of functional properties and viscosity behaviour of chia seed gel. *Journal of Pharmacognosy and Phytochemistry*, 8(3), 2969-2972. <https://www.phytojournal.com/archives/2019.v8.i3.8463/studies-on-extraction-and-characterization-of-functional-properties-and-viscosity-behavior-of-chia-seed-gel>
- Chaves, M. A., Piat, J., Malacarne, L. T., Gall, R. E., Colla, E., Bittencourt, P. R. S., de Souza, A. H. P., Gomes, S. T. M., & Matsushita, M. (2018). Extraction and application of chia mucilage (*Salvia hispanica* L.) and locust bean gum (*Ceratonia siliqua* L.) in goat milk frozen dessert. *Journal of Food Science and Technology*, 55(10), 4148-4158. <https://doi.org/10.1007/s13197-018-3344-2>
- Chemat, F., Zill-e-Huma, & Khan, M. K. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry*, 18(4), 813-835. <https://doi.org/10.1016/j.ultsonch.2010.11.023>
- Coorey, R., Tjoe, A., & Jayasena, V. (2014). Gelling properties of chia seed and flour: Chia gelling properties... *Journal of Food Science*, 79(5), E859-E866. <https://doi.org/10.1111/1750-3841.12444>
- de Campo, C., dos Santos, P. P., Costa, T. M. H., Paese, K., Guterres, S. S., Rios, A. de O., & Flôres, S. H. (2017). Nanoencapsulation of chia seed oil with chia mucilage (*Salvia hispanica* L.) as wall material: Characterisation and stability evaluation. *Food Chemistry*, 234, 1-9. <https://doi.org/10.1016/j.foodchem.2017.04.153>
- Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids*, 17(1), 25-39. [https://doi.org/10.1016/S0268-005X\(01\)00120-5](https://doi.org/10.1016/S0268-005X(01)00120-5)
- Farahmand, A., Naji Tabasi, S., & Shahbazizadeh, S. (2021). Influence of selected salts and sugars on the rheological behaviour of quince seed mucilage. *Journal of Agricultural Science and Technology*, 23(2), 333-347.
- Farahnaky, A., Bakhshizadeh-Shirazi, S., Mesbahi, G., Majzoobi, M., Rezvani, E., & Schleining, G. (2013). Ultrasound-assisted isolation of mucilaginous hydrocolloids from *Salvia macrosiphon* seeds and studying their functional properties. *Innovative Food Science & Emerging Technologies*, 20, 182-190. <https://doi.org/10.1016/j.ifset.2013.06.003>
- Hedayati, S., Niakousari, M., Babajafari, S., & Mazloomi, S. M. (2021). Ultrasound-assisted extraction of mucilaginous seed hydrocolloids: Physicochemical properties and food applications. *Trends in Food Science & Technology*, 118, 356-361. <https://doi.org/10.1016/j.tifs.2021.10.022>
- Ibañez, M. C., & Ferrero, C. (2003). Extraction and characterisation of the hydrocolloid from *Prosopis flexuosa* DC seeds. *Food Research International*, 36(5), 455-460. [https://doi.org/10.1016/S0963-9969\(02\)00192-8](https://doi.org/10.1016/S0963-9969(02)00192-8)
- Kar, F., & Arslan, N. (1999). Characterisation of orange peel pectin and effect of sugars, l-ascorbic acid, ammonium persulfate, salts on viscosity of orange peel pectin solutions. *Carbohydrate Polymers*, 40(4), 285-291. [https://doi.org/10.1016/S0144-8617\(99\)00063-6](https://doi.org/10.1016/S0144-8617(99)00063-6)
- Karazhiyan, H., Razavi, S. M. A., & Phillips, G. O. (2011). Extraction optimisation of a hydrocolloid extract from cress seed (*Lepidium sativum*) using response surface methodology. *Food Hydrocolloids*, 25(5), 915-920. <https://doi.org/10.1016/j.foodhyd.2010.08.022>
- Koocheki, A., Mortazavi, S. A., Shahidi, F., Razavi, S. M. A., & Taherian, A. R. (2009). Rheological properties of mucilage extracted from *Alyssum homolocarpum* seed as a new source of thickening agent. *Journal of Food Engineering*, 91(3), 490-496. <https://doi.org/10.1016/j.jfoodeng.2008.09.028>

- Koocheki, A., Taherian, A. R., & Bostan, A. (2013). Studies on the steady shear flow behavior and functional properties of *Lepidium perfoliatum* seed gum. *Food Research International*, 50(1), 446-456. <https://doi.org/10.1016/j.foodres.2011.05.002>
- Koocheki, A., Taherian, A. R., Razavi, S. M. A., & Bostan, A. (2009). Response surface methodology for optimisation of extraction yield, viscosity, hue and emulsion stability of mucilage extracted from *Lepidium perfoliatum* seeds. *Food Hydrocolloids*, 23(8), 2369-2379. <https://doi.org/10.1016/j.foodhyd.2009.06.014>
- Lin, H.-Y., & Lai, L.-S. (2009). Isolation and viscometric characterisation of hydrocolloids from mulberry (*Morus alba* L.) leaves. *Food Hydrocolloids*, 23(3), 840-848. <https://doi.org/10.1016/j.foodhyd.2008.03.018>
- Maran, J. P., & Priya, B. (2014). Ultrasound-assisted extraction of polysaccharide from *Nephelium lappaceum* L. fruit peel. *International Journal of Biological Macromolecules*, 70, 530-536. <https://doi.org/10.1016/j.ijbiomac.2014.07.032>
- Marcotte, M., Taherian, A. R., Trigui, M., & Ramaswamy, H. S. (2001). Evaluation of rheological properties of selected salt enriched food hydrocolloids. *Journal of Food Engineering*, 48(2), 157-167. [https://doi.org/10.1016/S0260-8774\(00\)00153-9](https://doi.org/10.1016/S0260-8774(00)00153-9)
- Morales-Tovar, M. E., Ramos-Ramírez, E. G., & Salazar-Montoya, J. A. (2020). Modelling and optimisation of the parameters affecting extraction of the chan seed mucilage (*Hyptis suaveolens* (L.) Poit) by mechanical agitation (MA) and ultrasound-assisted extraction (UAE) in a multiple variables system. *Food and Bioprocess Processing*, 120, 166-178. <https://doi.org/10.1016/j.fbp.2020.01.009>
- Muñoz, L. A., Cobos, A., Diaz, O., & Aguilera, J. M. (2012). Chia seeds: Microstructure, mucilage extraction and hydration. *Journal of Food Engineering*, 108(1), 216-224. <https://doi.org/10.1016/j.jfoodeng.2011.06.037>
- Orifici, S. C., Capitani, M. I., Tomás, M. C., & Nolasco, S. M. (2018). Optimisation of mucilage extraction from chia seeds (*Salvia hispanica* L.) using response surface methodology. *Journal of the Science of Food and Agriculture*, 98(12), 4495-4500. <https://doi.org/10.1002/jsfa.8974>
- Pérez-Orozco, J. P., Sánchez-Herrera, L. M., & Ortiz-Basurto, R. I. (2019). Effect of concentration, temperature, pH, co-solutes on the rheological properties of *Hyptis suaveolens* L. mucilage dispersions. *Food Hydrocolloids*, 87, 297-306. <https://doi.org/10.1016/j.foodhyd.2018.08.004>
- Salehi, F., Kashaninejad, M., & Behshad, V. (2014). Effect of sugars and salts on rheological properties of balangu seed (*Lallemantia royleana*) gum. *International Journal of Biological Macromolecules*, 67, 16-21. <https://doi.org/10.1016/j.ijbiomac.2014.03.001>
- Saporittis, K., Marasco, J., Morales, R., Martinez, M. J., & Pilosof, A. M. (2023). Unravelling the relationship between conditions of ultrasonic-assisted extraction of chia mucilage and viscosity. *Journal of the Science of Food and Agriculture*, 103(8), 3860-3870. <https://doi.org/10.1002/jsfa.12300>
- Saqib, M. N., Khaled, B. M., Liu, F., & Zhong, F. (2022). Hydrogel beads for designing future foods: Structures, mechanisms, applications, and challenges. *Food Hydrocolloids for Health*, 2, Article 100073. <https://doi.org/10.1016/j.fhfh.2022.100073>
- Silva, L. A., Sinnecker, P., Cavalari, A. A., Sato, A. C. K., & Perrechil, F. A. (2022). Extraction of chia seed mucilage: Effect of ultrasound application. *Food Chemistry Advances*, 1, Article 100024. <https://doi.org/10.1016/j.focha.2022.100024>

- Szczesniak, A. S., & Farkas, E. (1962). Objective characterisation of the mouthfeel of gum solutions. *Journal of Food Science*, 27(4), 381-385. <https://doi.org/10.1111/j.1365-2621.1962.tb00112.x>
- Taherian, A. R., Fustier, P., & Ramaswamy, H. S. (2007). Effects of added weighting agent and xanthan gum on stability and rheological properties of beverage cloud emulsions formulated using modified starch. *Journal of Food Process Engineering*, 30(2), 204-224. <https://doi.org/10.1111/j.1745-4530.2007.00109.x>
- Timilsena, Y. P., Adhikari, R., Kasapis, S., & Adhikari, B. (2015). Rheological and microstructural properties of the chia seed polysaccharide. *International Journal of Biological Macromolecules*, 81, 991-999. <https://doi.org/10.1016/j.ijbiomac.2015.09.040>
- Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., López Malo Vigil, A., & Liceaga, A. M. (2019). Physicochemical characteristics of chia seed (*Salvia hispanica*) protein hydrolysates produced using ultrasonication followed by microwave-assisted hydrolysis. *Food Hydrocolloids*, 97, Article 105187. <https://doi.org/10.1016/j.foodhyd.2019.105187>
- Vardhanabhuti, B., & Ikeda, S. (2006). Isolation and characterisation of hydrocolloids from monoi (*Cissampelos pareira*) leaves. *Food Hydrocolloids*, 20(6), 885-891. <https://doi.org/10.1016/j.foodhyd.2005.09.002>
- Yanes, M., Durán, L., & Costell, E. (2002). Effect of hydrocolloid type and concentration on flow behaviour and sensory properties of milk beverages model systems. *Food Hydrocolloids*, 16(6), 605-611. [https://doi.org/10.1016/S0268-005X\(02\)00023-1](https://doi.org/10.1016/S0268-005X(02)00023-1)
- Yang, D., Gao, S., & Yang, H. (2020). Effects of sucrose addition on the rheology and structure of iota-carrageenan. *Food Hydrocolloids*, 99, Article 105317. <https://doi.org/10.1016/j.foodhyd.2019.105317>
- Yousefi, A. R., Razavi, S. M. A., & Khodabakhsh Aghdam, S. H. (2014). Influence of temperature, mono- and divalent cations on dilute solution properties of sage seed gum. *International Journal of Biological Macromolecules*, 67, 246-253. <https://doi.org/10.1016/j.ijbiomac.2014.03.026>
- Zia, S., Khan, M. R., Shabbir, M. A., Aslam Maan, A., Khan, M. K. I., Nadeem, M., Khalil, A. A., Din, A., & Aadil, R. M. (2022). An inclusive overview of advanced thermal and nonthermal extraction techniques for bioactive compounds in food and food-related matrices. *Food Reviews International*, 38(6), 1166-1196. <https://doi.org/10.1080/87559129.2020.1772283>

Review Article

Red Ginger's Tissue Culture to Improve the Production of Phenolic Compounds Related to Antioxidant and Antibacterial Activity

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ABSTRACT

Phenolic compounds are one of the highest secondary metabolites in red ginger (*Zingiber officinale* var. *rubrum*). The most abundant and powerful phenolic compound is 6-gingerol, which is responsible for the pungent effect in red ginger. Unfortunately, the production of phenolic compounds is influenced by many factors in field-grown red ginger. Hence, it needs a more appropriate technique to produce a higher amount of phenolic compounds. One of the potential techniques is plant tissue culture, which has been known as an efficient technique to grow valuable plants in vitro to generate optimal production of secondary metabolites in controlled aseptic conditions. This study aims to investigate how tissue culture provides the enhancement of phenolic compounds production, related

to antioxidant and antibacterial activity. It was expected to provide the defence response of plants due to the treatment added, like hormone, elicitors, manipulation of abiotic conditions, etc. In red ginger, the callus extract showed higher total phenolic content than the rhizomes. The addition of elicitors like yeast extract and salicylic acid succeeded in increasing total phenolic content in ginger. The higher total phenolic content in tissue culture-cultivated red ginger was expected to contribute to higher antioxidant and antibacterial activity. So, plant

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tissue culture is assumed to facilitate efficient production of secondary metabolites, including phenolic compounds, although its relation to antioxidant and antibacterial activity is still unclear. It may need some further research to prove a direct link between the enhancement of phenolic, antioxidant and antibacterial activity in red ginger's tissue culture.

Keywords: Antibacterial, elicitor, phenol, red ginger, tissue culture

INTRODUCTION

Red Ginger's Cultivation

Ginger (*Zingiber officinale*) is a plant widely used in Asia as a spice and herbal medicine (Suciwati & Adnyana, 2017; Widyastuti et al., 2024a; Zhang et al., 2022) for thousands of years (Miri, 2020). Based on the plant database in www.theplantlist.org (accessed on October 18th, 2024), there are eight names for the species of ginger, of which one is the accepted name and the other seven are synonyms. The accepted name is *Zingiber officinale* Roscoe, and the synonyms are *Z. officinale* var. *cholmondeleyi* F. M. Bailey; *Z. officinale* f. *macrorrhizonum* (Makino) M. Hiroe; *Z. officinale* var. *macrorrhizonum* Makino; *Z. officinale* f. *rubens* (Makino) M. Hiroe; *Z. officinale* var. *rubens* Makino; *Z. officinale* var. *rubrum* Theilade; and *Z. officinale* var. *sichuanense* (Z. Y. Zhu, S. L. Zhang & S. X. Chen).

Commonly, gingers are categorised into three varieties based on the differences in size and colour of their rhizomes. There are white giant ginger (*Z. officinale* Rosc. var. *officinale*), small white ginger (*Z. officinale* Rosc. var. *amarum*), and red ginger (*Z. officinale* Rosc. var. *rubrum*) (Zhang et al., 2022). All three varieties are reproductively sterile. They are only propagated via their rhizomes, vegetatively. However, the vegetative cultivated red ginger is susceptible to *Ralstonia solanacearum*, *Pythium* spp., *Fusarium* spp., and some other soil microbes, which cause the rhizome rot (Miri, 2020). Red ginger, in some places known as “sunti” ginger. Some synonyms of red ginger's scientific names are *Z. officinale* Roscoe var. *sunti* Val., *Z. amomum* L., *Z. cholmondeleyi* (F. M. Bailey) K. Schum, *Z. missionis* Wall., and *Z. sichuanense* (Nurdyansyah & Widyastuti, 2022).

Red ginger has red-coloured rhizomes, different from the two other varieties, which have white-coloured rhizomes. The red colour is related to its anthocyanin content (Widyastuti et al., 2024a). Red ginger is included in the division of Spermatophyta, subdivision of Angiospermae, class of Monocotyledoneae, order of Zingiberales, and Zingiberaceae family. It is mainly cultivated in Asia (Suciwati & Adnyana, 2017), especially in China, Indonesia, and Malaysia (Zhang et al., 2022). The conventional cultivation of red ginger uses its rhizome to grow a new plant. However, it possesses some weaknesses such as low proliferation rate, long growth time, and soil pathogen infection resulting in damage to the damage in rhizome. Red ginger cultivation has to face the limitation of superior seeds, so it may need other techniques to cultivate red ginger (Nurdyansyah & Widyastuti, 2022).

The selection of rhizomes to use as seeds for conventional cultivation has to be done. Big fresh rhizomes with no wrinkles and no scars are usually used as seeds to cultivate red ginger vegetatively. Red ginger's cultivation mostly is in the end of the rainy season to avoid decomposition due to high rain intensity. To maintain the growth of red ginger, the plant has to be watered, fertilised, and weeded. Red ginger can be harvested after 3 to 4 months of planting (Nurdyansyah & Widyastuti, 2022).

Red Ginger's Bioactive Compounds

Red ginger contains many bioactive compounds with different biological effects. Its essential oils and oleoresins produce a strong, pungent, and sour flavour. Fresh rhizomes possess lipids (0.9%), minerals (1.2%), proteins (2.3%), fibre (2.4%), carbohydrates (12.3%), and water (80.9%) (Beristain-Bauza et al., 2019). It has antioxidant agents which correlated to its phenolic and flavonoid contents. Some phenolic compounds known in red ginger are 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, 10-shogaol (Figure 1), paradol, and zingeron. On the other hand, it is already well-reported that all varieties of ginger also contain a large amount of flavonoids, which are natural antioxidants and can reduce the risk of heart disease, cancer, and high blood pressure (Jiang et al., 2017). Some flavonoid compounds known are rutin, quercetin, catechin, and epicatechin (Widyastuti et al., 2024a).

Red ginger possesses about 169 bioactive compounds, including monoterpenes, sesquiterpenes, diterpenes, flavonoids, phenolic compounds, vanilloids, etc. Moreover, it also contains vitamins, amino acids, and some trace elements such as copper, iron, zinc, chromium, manganese, nickel, etc (Zhang et al., 2022). Total phenolic and flavonoid content in red ginger is known to be higher than that of white ginger (Ghasemzadeh et al., 2010). Hundreds of its bioactive compounds were classified into three categories: gingerols, volatile oils, and diarylheptanoids. Gingerols belong to the phenols that include gingerone, shogaols, and paradols. Whereas, the volatile oils include monoterpenoids and sesquiterpenoids. The diarylheptanoids are a class of derivatives which have a 1,7-diarylheptane skeleton (Jiang et al., 2017).

The 6-gingerol is the most abundant and plays a role as the most powerful pharmacologically active phenolic compound found in the rhizomes of red ginger (Widyastuti et al., 2025). While the other gingerols, like 4-, 8-, 10, and 12-gingerols, are found in lower concentrations (Beristain-Bauza et al., 2019). This 6-gingerol possesses some biological activities such as anti-inflammatory, antioxidant, anti-mutagenic, anti-carcinogenic, etc. 6-gingerol is also known to have a therapeutic effect in osteoarthritis by attenuating the oxidative stress as well as downregulating the pro-inflammatory mediators. It also inhibits the metastasis and induces cell death in cancer cell lines (Alsahli et al., 2021).

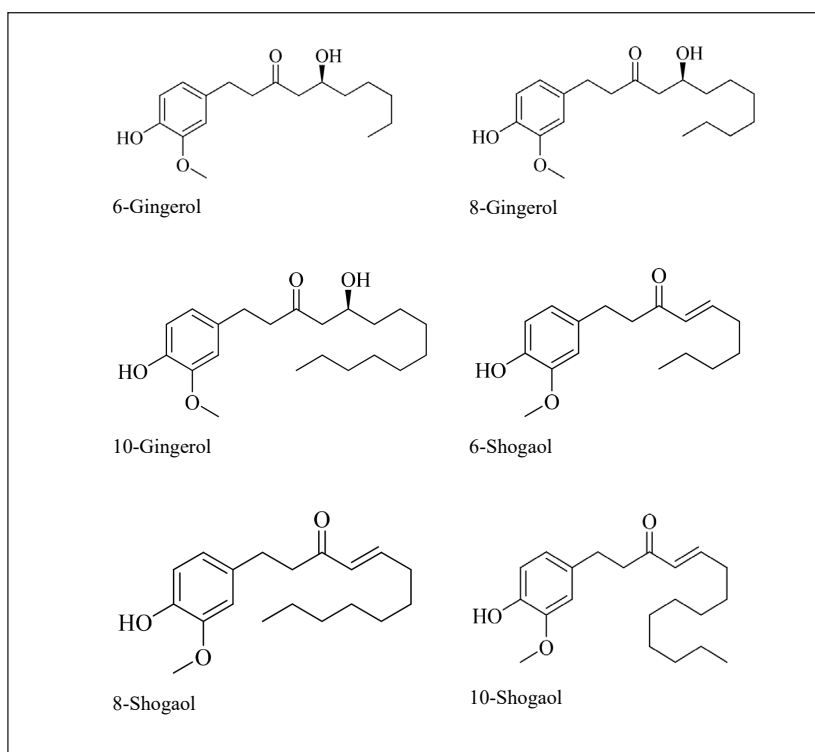


Figure 1. The main phenolic compounds in red ginger (Zhang et al., 2022)

Gingerols, including 4-, 6-, 8-, 10-, and 12-gingerols, are dominant in fresh rhizomes of red ginger. However, the 6-, 8-, and 10-shogaols are dominant in dry rhizomes (Hossain et al., 2019; Zhang et al., 2022). Gingerols and their derivatives play an important role as pungent bioactive compounds (Beristain-Bauza et al., 2019; Yudthavorasit et al., 2014). Based on GC-MS results, red ginger oils contain three monoterpenes (14.5% champene, 14.3% geranial, and 13.7% geranyl acetate) and 47 sesquiterpenes.

PLANT TISSUE CULTURE FOR RED GINGER'S CULTIVATION

Benefits of Plant Tissue Culture

Plant tissue culture is a technique to optimise plant production by micropropagation, genetic transformation, gene modification, germplasm preservation, pathogen eradication, etc. This technique is an important tool in agriculture, industry, and plant breeding, etc (Custódio et al., 2023). Plant tissue and organ culture can be described as the manipulation of cells and/or organs in aseptic conditions, grown in a suitable culture medium, and under controlled conditions like temperature, humidity, light, etc (Dias et al., 2016). It uses culture media

with some nutrients and hormones, controlled aseptic conditions, and optimal abiotic conditions for the growth of cells, tissues, and organs.

Plant tissue and organ culture can be initiated from any part of the plant based on its totipotency. For example, the shoot tip is known as an ideal explant for micropropagation or direct shoot regeneration (Bhoite & Palshikar, 2014). Plant tissue culture supports rapid multiplication of rare plant genotypes, plant genome transformation or modification, and production of disease-free plants in a shorter time (Mehaboob et al., 2019), and production of important plant-derived secondary metabolites (Espinosa-Leal et al., 2018). Furthermore, the addition of plant growth hormones (synthetic or natural phytohormones) can manipulate the plant development (Phillips & Garda, 2019). The rare plant species which economically important can be produced in larger quantities through tissue culture (Bhoite & Palshikar, 2014).

Plant tissue culture is also an efficient alternative technique to manipulate the production of secondary metabolites due to the response to different types of stress given in the culture (Winson et al., 2021). Plant secondary metabolites are mainly used across industries, such as cosmetics, pharmaceuticals, fragrances, supplements, dyes, or flavours. The biochemical properties extracted from plant tissue cultures are also easier to purify, so they can reduce the cost of production (Babu et al., 2020; Humbal & Pathak, 2023). So, the plant tissue culture is one of the promising techniques to meet the growing demands of plant secondary metabolites (Ramirez-Estrada et al., 2016). This technique allows better control of the plant development, production of some specific tissues, and also the activation of biochemical pathway which trigger the synthesis of specific secondary metabolites (Cardoso et al., 2019).

Plant Tissue Culture to Enhance Red Ginger's Bioactive Compounds

The secondary metabolites production of plants usually comes in a low concentration (< 1% dry weight) due to environmental factors (Winson et al., 2021). Plant tissue culture emerges as a viable biotechnological technique for the production of important secondary metabolites of plants, which can be used in the pharmaceutical or biological function (Espinosa-Leal et al., 2018). Cultivation of plant cells through in vitro tissue culture could be potentially competitive for the effective production of secondary metabolites. The number of phytohormones added plays a vital role in the enhancement of callus growth, leading to the enhancement of secondary metabolites production (Kumari et al., 2016).

The accumulation of plant secondary metabolites is a defence mechanism against unstable conditions, like abiotic stresses, pathogen attacks, bacterial or fungal infections, or others (Mathur, 2018). With plant tissue culture, the production of plant secondary metabolites can be improved by the treatment of elicitors (Ali et al., 2018), both biotic or abiotics elicitor, which can activate the signal compounds of plant defence responses (Mathur, 2018). The addition of elicitors can overcome the limitations of plant tissue culture,

Elicitation is a strategy in plant tissue culture to enhance the production of bioactive compounds through activating the plant defence mechanism for scavenging the ROS (reactive oxygen species). The activation of genes to produce various enzymes that are involved in plant defence mechanisms will modulate the production of secondary metabolites related to those defence responses (Bakhtiari & Golkar, 2022). The way of elicitor application (Złotek et al., 2019), duration, concentration, and nature of elicitor (Figure 3) are some important influencing factors to induce the optimal production of secondary metabolites (Al-Khayri & Naik, 2020). The elicitation in ginger's cultures was proven to show a significant escalation of antioxidant activity, compared to the unelicited culture (Ali et al., 2018). Elicitation can encounter the limitations of low secondary metabolite production in plant tissue culture. It generates higher amounts of specialised compounds compared to others with no elicitors. The use of biotic or abiotic elicitors stimulates molecular responses in plant tissue cultures, which lead to the enhancement of secondary metabolites production (Arya et al., 2022).

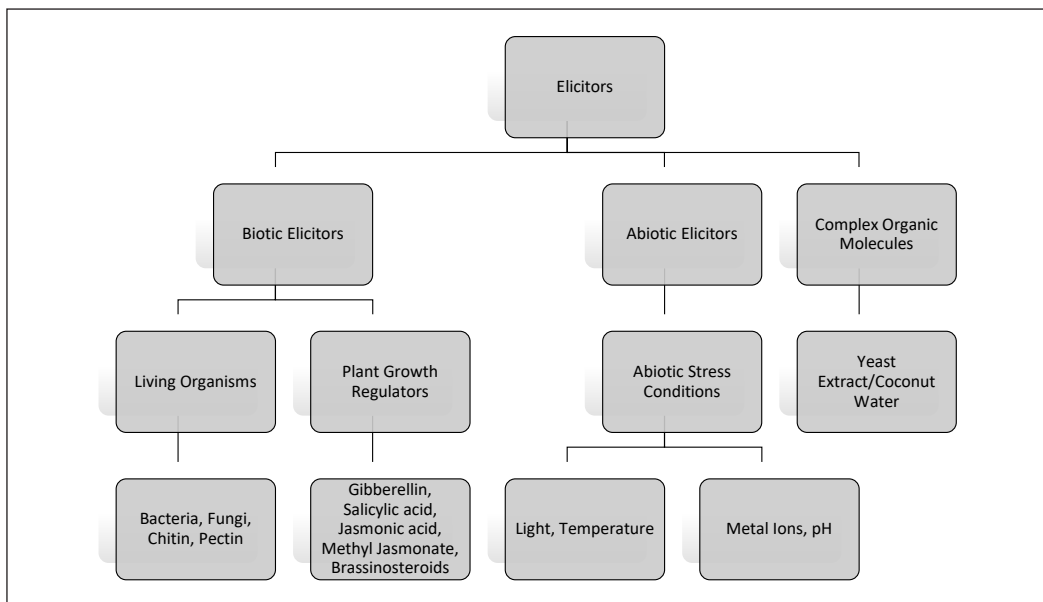


Figure 3. A graphical classification of classes of elicitors (Nabi et al., 2021)

PRODUCTION OF PHENOLIC COMPOUNDS IN RED GINGER

Enhancement of Phenolic Compounds with Plant Tissue Culture

All varieties of ginger are known as plants with high phenolic compounds (Widayat et al., 2018). The total phenolic compounds in red ginger have a positive correlation to its antioxidant activities. The high level of total phenolic and flavonoid content indicated high

antioxidant activities in Halia Bara ginger (Ghafoor et al., 2020; Johari & Khong, 2019). Unfortunately, the production of phenolic compounds is decreased with elevation (Nataraj et al., 2022). So, in red ginger, it is quite challenging to take advantage of its phenolic compounds like gingerol and shogaol from field-grown rhizomes. It requires a large amount of red ginger's rhizomes to meet the needs of the pharmaceutical and industrial sectors, which leads to bigger production costs.

The phenolics are usually found in limited amounts in naturally grown plants, and their biosynthesis is sensitive to stress conditions, both biotic and abiotic stresses, like pathogen-induced damage, extreme temperatures, nutritional deficiencies, drought, etc. Hence, plant tissue culture offers a reliable system to provide phenolic biosynthesis under controlled conditions (García-Pérez et al., 2020).

Plant tissue culture has been known as an effective technique to improve the growth and metabolism rates. The culture media support cell growth initiation to faster cell proliferation, so that the secondary metabolites production will also increase. Red ginger with a low proliferation rate can utilise this technique to optimisation of secondary metabolites production (Widyastuti et al., 2024a). The use of plant tissue culture on Murashige and Skoog (MS) media with a high concentration of sucrose (about 60-90 gL⁻¹) promotes the differentiation of microrhizomes in ginger culture. Furthermore, optimised levels of hormones like NAA and 2,4-D promote the calli production of ginger culture (Rani et al., 2022). The plant tissue culture is also expected to produce secondary metabolites at lower costs (Mamdouh & Smetanska, 2022).

To increase the production of phenolic compounds in red gingers, the rhizome's buds can be used as an explant to induce callus. The callus culture can lead to increasing of phenolic compounds production. Furthermore, cell suspension cultures also maintain cells in suspensions, which enhances the synthesis of phenolics by providing optimal growth conditions. Applying biotics or abiotics elicitors is also known to stimulate the higher production of phenolic compounds in red ginger's tissue cultures. *Aspergillus niger* and *Fusarium oxysporum* were known to be used as biotic elicitors in *Echinacea purpurea* callus and succeeded in enhancing its production of phenolics, flavonoids, and antioxidant capacity (Elshahawy et al., 2022). The use of 0.1% yeast extract and 10 µM jasmonic acids was proven to increase the total phenolic content in lovage leaves (*Levisticum officinale* Koch) cultures (Złotek et al., 2019).

In white ginger tissue culture, the treatment of callus with elicitors like yeast extract, salicylic acid, and glycine was proven to significantly enhance the total phenolic content (Ali et al., 2018). The development of red ginger's callus in MS media supplemented with 2,4-D and Kinetin generates higher total phenolic content than rhizomes (Widyastuti et al., 2024b). Cell suspension culture of Sabah snake grass (*Clinacanthus nutans*) generates higher total phenolic content than leaf and steam extracts (Bong et al., 2021). The callus

induction of *Nepeta binaloudensis* in ½ MS media supplemented with the combination of BAP and NAA has been known to produce optimal concentrations of secondary metabolites like phenolic, flavonoid, and tannin (Sagharyan et al., 2020). *Lepidium sativum* callus elicited with 250 mg/L chitosan performs higher accumulations of total phenolics, phenylalanine (PAL) activity, reactive scavenging ability (RSA), and relative callus fresh weight (Bakhtiari & Golkar, 2022).

Phenolic Compounds Related to Antioxidant and Antibacterial Activity

The phenolic compounds are molecules which possess at least one phenol unit. It can be obtained from marine organisms, bacteria, fungi, but mostly from plants. Phenolic compounds are classified into some subcategories based on their chemical structure, including simple phenols, phenolic acids, flavonoids, tannins, coumarins, lignans, curcuminoids, quinones, and stilbenes (Ecevit et al., 2022). Polyphenols have aromatic carboxylic acids with multiple phenolic hydroxyl groups, which are substituted on one ring of benzene with concomitant features (Fu et al., 2020). Polyphenols reflect the most general phytochemicals with various beneficial health effects. Phenolics have some biological activities, from antioxidants to antiproliferative compounds (Lobiuc et al., 2023).

The phenolic acids derived from plants have been reported to have antioxidant, antimicrobial, anti-inflammatory, and pro-coagulant properties. It provided some antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* at appropriate concentrations that were possible for integration into polymeric biomaterials (Liu et al., 2020). Phenolic compounds derived from plants have demonstrated antibacterial properties against some foodborne pathogens, like *Salmonella* sp. (Widyastuti et al., 2026), *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, and *E. coli*. Phenols synergise with antimicrobials in clinical matters to increase the antimicrobial effectiveness and mitigate resistance in foodborne pathogens (Padilha da Silva et al., 2024). A type of phenol, gallic acid extracted from *Hibiscus sabdariffa*, needs 50 mg/L to kill *Salmonella enterica* serovar Typhimurium (SeleneMárquez-Rodríguez et al., 2020).

The antioxidant potential contributes to the antibacterial activity as well as free radical scavenging activity (Pradhan et al., 2021). Phenolic acids have antimicrobial properties correlated to their hydrogen-donating groups, which could be reactive to oxidants, then form the resonance-stabilised phenoxyl radicals. Those will provide antioxidant activity to improve healing outcomes. Phenolic acids are shown as multifunctional molecules to improve the healing outcomes and reduce drug-resistant infections. However, they were commonly studied in relation to foodborne illness and plant pathogens. The study about antimicrobial properties of phenolic acids in clinical bacterial infections and wound bacteria is just limited (Liu et al., 2020).

Red ginger with its anthocyanin has the structure of flavylum cation (AH^+), which correlates to its antioxidant activity (Widyastuti et al., 2024a). Anthocyanins are one of the phenolic compounds that are characterised as the largest group of water-soluble pigments. They have various biological activities, including their ability to deactivate reactive oxygen species (ROS) as one of the free radicals, improve the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities as antioxidant enzymes, increase glutathione content, and reduce the DNA oxidative damage (Abdurrahim et al., 2023). Those activities mean that the presence of anthocyanins in red ginger can increase its antioxidant and antibacterial activities. The combination of gingerols and anthocyanins in red ginger was estimated to trigger a synergistic antioxidant effect against oxidative stress (Abdurrahim et al., 2023).

The phenolic acids have antibacterial activity against Gram-positive and Gram-negative bacteria. The Gram-positive bacteria are most sensitive to phenolic compounds due to their peptidoglycans, which leads to the lack of an outer membrane. However, their antibacterial activity depends on the bacterial strains, species (Lobiuc et al., 2023), and the chemical structure of compounds, like chain length, number of benzene rings, and position of substituents on the core of benzene rings. The phenolic acids show bactericidal actions, resulting in bacterial cell death (Ecevit et al., 2022). The undissociated forms of phenolic acids cross through cell membranes with passive diffusion, then acidifying the cytoplasm and causing the outflow of the essential intracellular constituents of the cell, including nucleic acids, proteins, and inorganic ions like phosphate or potassium (Lobiuc et al., 2023), resulting in bacterial cell death (Ecevit et al., 2022). The phenolics have several mechanisms to kill the bacterial cells (Figure 4), including blocking the enzymatic activity

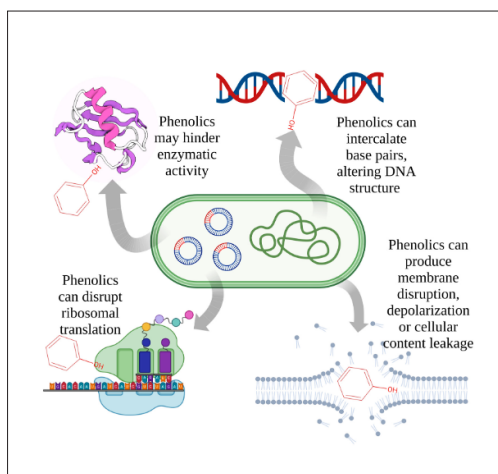


Figure 4. The mechanisms of antibacterial activity of phenolic compounds (Lobiuc et al., 2023)

of the bacterial cells, inserting base pairs or altering the DNA structure, interrupting the ribosomal translation, and producing cell membrane disruption, depolarisation, or cellular leakage (Lobiuc et al., 2023).

The enhancement of phenolic compounds production of red ginger through plant tissue culture provides the improvement of its antibacterial activity. The phenolic compounds in blackcurrant have been proven to have antibacterial activity against *E. coli*, *S. aureus*, and *Salmonella* sp. Furthermore, the anthocyanin in blackcurrant, just like in red ginger, showed moderate antibacterial

activity against *E. coli* and *S. aureus* (Zhao et al., 2021). With the higher total phenolic content (Widyastuti et al., 2024b) and antioxidant activities in red ginger callus through *in vitro* cultivation, it might also be predicted to have antibacterial activity against several bacterial strains.

CONCLUSION

Plant tissue culture is known to be an alternative cultivation for vegetatively weak red ginger. This technique allows for increased production of secondary metabolites with other characteristics compared to fieldgrown red gingers. The enhancement of secondary metabolite production can be affected by some factors, such as biotic and abiotic conditions, addition of hormones, treatment of elicitors, etc. The red ginger's callus extract has been known to have higher total phenolic content than the field-grown rhizome's extract. The elicitors like yeast extract and salicylic acid are known to generate higher total phenolic content and antioxidant activity of red ginger's callus, which may lead to higher antibacterial activity. Furthermore, the relation among phenolic, antioxidant, and antibacterial activity may be synergistically interconnected, but there are possibilities that the antioxidant and antibacterial activity are not only related to total phenolic content but also to other internal or external factors of the cultures.

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REFERENCES

- Abdurrahim, A. E., Mazurak, V. C., & Chen, L. (2023). Gingerols synergise with anthocyanins to induce antioxidant activity in vitro. *Frontiers in Nutrition*, 10, Article 1229015. <https://doi.org/10.3389/fnut.2023.1229015>
- Ali, A. M. A., El-Nour, M. E. A. M., & Yagi, S. M. (2018). Total phenolic and flavonoid contents and antioxidant activity of ginger (*Zingiber officinale* Rosc.) rhizome, callus and callus treated with some elicitors. *Journal of Genetic Engineering and Biotechnology*, 16(2), 677-682. <https://doi.org/10.1016/j.jgeb.2018.03.003>
- Al-Khayri, J. M., & Naik, P. M. (2020). Elicitor-induced production of biomass and pharmaceutical phenolic compounds in cell suspension culture of date palm (*Phoenix dactylifera* L.). *Molecules*, 25(20), Article 4669. <https://doi.org/10.3390/molecules25204669>
- Alsahli, M. A., Almatroodi, S. A., Almatroudi, A., Khan, A. A., Anwar, S., Almutary, A. G., Alrumaihi, F., & Rahmani, A. H. (2021). 6-Gingerol, a major ingredient of ginger attenuates diethylnitrosamine-induced liver injury in rats through the modulation of oxidative stress and anti-inflammatory activity. *Mediators of Inflammation*, 2021(1), Article 6661937. <https://doi.org/10.1155/2021/6661937>

- Arya, S. S., Rookes, J. E., Cahill, D. M., & Lenka, S. K. (2022). Chitosan nanoparticles and their combination with methyl jasmonate for the elicitation of phenolics and flavonoids in plant cell suspension cultures. *International Journal of Biological Macromolecules*, 214, 632-641. <https://doi.org/10.1016/j.ijbiomac.2022.06.145>
- Babu, N. G. R., Suresh, M., Zavith, M., Boopathi, R., & Parvathy, S. (2020). Standardisation of ginger callus culture and its comparison with ginger rhizome and callus treated with extrinsic molecule on their antioxidant activity. *International Journal of Herbal Medicine*, 8(4), 49-54. <https://www.florajournal.com/archives/2020/vol8issue4/PartA/7-5-11-327.pdf>
- Bakhtiari, M. A., & Golkar, P. (2022). The effects of callus elicitation on lepidine, phenolic content, and antioxidant activity of *Lepidium sativum* L.: Chitosan and gibberellic acid. *Journal of Plant Growth Regulation*, 41(3), 1148-1160. <https://doi.org/10.1007/s00344-021-10368-5>
- Beristain-Bauza, S. D. C., Hernández-Carranza, P., Cid-Pérez, T. S., Ávila-Sosa, R., Ruiz-López, I. I., & Ochoa-Velasco, C. E. (2019). Antimicrobial activity of ginger (*Zingiber officinale*) and its application in food products. *Food Reviews International*, 35(5), 407-426. <https://doi.org/10.1080/87559129.2019.1573829>
- Bhoite, H. A., & Palshikar, G. S. (2014). Plant tissue culture: A review. *World Journal of Pharmaceutical Sciences*, 2(6), 565-572. <http://www.wjpsonline.org/>
- Bong, F. J., Yeou Chear, N. J., Ramanathan, S., Mohana-Kumaran, N., Subramaniam, S., & Chew, B. L. (2021). The development of callus and cell suspension cultures of Sabah snake grass (*Clinacanthus nutans*) for the production of flavonoids and phenolics. *Biocatalysis and Agricultural Biotechnology*, 33, Article 101977. <https://doi.org/10.1016/j.bcab.2021.101977>
- Cardoso, J. C., de Oliveira, M. E. B. S., & Cardoso, F. de C. I. (2019). Advances and challenges on the in vitro production of secondary metabolites from medicinal plants. *Horticultura Brasileira*, 37(2), 124-132. <https://doi.org/10.1590/s0102-053620190201>
- Custódio, L., Charles, G., Magné, C., Barba-Espín, G., Piqueras, A., Hernández, J. A., Ben Hamed, K., Castañeda-Loaiza, V., Fernandes, E., & Rodrigues, M. J. (2023). Application of in vitro plant tissue culture techniques to halophyte species: A review. *Plants*, 12(1), Article 126. <https://doi.org/10.3390/plants12010126>
- Dias, M. I., Sousa, M. J., Alves, R. C., & Ferreira, I. C. F. R. (2016). Exploring plant tissue culture to improve the production of phenolic compounds: A review. *Industrial Crops and Products*, 82, 9-22. <https://doi.org/10.1016/j.indcrop.2015.12.016>
- Ecevit, K., Barros, A. A., Silva, J. M., & Reis, R. L. (2022). Preventing microbial infections with natural phenolic compounds. *Future Pharmacology*, 2(4), 460-498. <https://doi.org/10.3390/futurepharmacol2040030>
- Elshahawy, O. A. M., Zeawail, M. E. F., Hamza, M. A., Elateeq, A. A., & Omar, M. A. (2022). Improving the production of total phenolics and flavonoids and the antioxidant capacity of *Echinacea purpurea* callus through biotic elicitation. *Egyptian Journal of Chemistry*, 65(12), 137-149. <https://doi.org/10.21608/ejchem.2022.145210.6328>
- Espinosa-Leal, C. A., Puente-Garza, C. A., & García-Lara, S. (2018). In vitro plant tissue culture: Means for production of biological active compounds. *Planta*, 248, 1-18. <https://doi.org/10.1007/s00425-018-2910-1>

- Fazili, M. A., Bashir, I., Ahmad, M., Yaqoob, U., & Geelani, S. N. (2022). In vitro strategies for the enhancement of secondary metabolite production in plants: A review. *Bulletin of the National Research Centre*, 46, Article 35. <https://doi.org/10.1186/s42269-022-00717-z>
- Fu, R., Shi, M., Deng, C., Zhang, Y., Zhang, X., Wang, Y., & Kai, G. (2020). Improved phenolic acid content and bioactivities of *Salvia miltiorrhiza* hairy roots by genetic manipulation of RAS and CYP98A14. *Food Chemistry*, 331, Article 127365. <https://doi.org/10.1016/j.foodchem.2020.127365>
- García-Pérez, P., Lozano-Milo, E., Landín, M., & Gallego, P. P. (2020). Combining medicinal plant in vitro culture with machine learning technologies for maximising the production of phenolic compounds. *Antioxidants*, 9(3), Article 210. <https://doi.org/10.3390/antiox9030210>
- Ghafoor, K., Al Juhaimi, F., Özcan, M. M., Uslu, N., Babiker, E. E., & Mohamed Ahmed, I. A. (2020). Total phenolics, total carotenoids, individual phenolics and antioxidant activity of ginger (*Zingiber officinale*) rhizome as affected by drying methods. *Lwt*, 126, Article 109354. <https://doi.org/10.1016/j.lwt.2020.109354>
- Ghasemzadeh, A., Jaafar, H. Z. E., & Rahmat, A. (2010). Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysian young ginger (*Zingiber officinale* Roscoe). *Molecules*, 15(6), 4324-4333. <https://doi.org/10.3390/molecules15064324>
- Hossain, S., De Silva, B. C. J., Wimalasena, S. H. M. P., Pathirana, H. N. K. S., & Heo, G. J. (2019). In vitro antibacterial effect of ginger (*Zingiber officinale*) essential oil against fish pathogenic bacteria isolated from farmed olive flounder (*Paralichthys olivaceus*) in Korea. *Iranian Journal of Fisheries Sciences*, 18(2), 386-394.
- Humbal, A., & Pathak, B. (2023). Harnessing nanoparticle-mediated elicitation in plant tissue culture: A promising approach for secondary metabolite production. *Plant Cell, Tissue and Organ Culture*, 155(2), 385-402. <https://doi.org/10.1007/s11240-023-02612-5>
- Jiang, Y., Liao, Q., Zou, Y., Liu, Y., & Lan, J. (2017). Transcriptome analysis reveals the genetic basis underlying the biosynthesis of volatile oil, gingerols, and diarylheptanoids in ginger (*Zingiber officinale* Rosc.). *Botanical Studies*, 58, Article 41. <https://doi.org/10.1186/s40529-017-0195-5>
- Johari, M. A., & Khong, H. Y. (2019). Total phenolic content and antioxidant and antibacterial activities of *Pereskia bleo*. *Advances in Pharmacological Sciences*, 2019, Article 428593. <https://doi.org/10.1155/2019/7428593>
- Kumari, A., Baskaran, P., & Van Staden, J. (2016). In vitro propagation and antibacterial activity in *Cotyledon orbiculata*: A valuable medicinal plant. *Plant Cell, Tissue and Organ Culture*, 124(1), 97-104. <https://doi.org/10.1007/s11240-015-0878-0>
- Liu, J., Du, C., Beaman, H. T., & Monroe, M. B. B. (2020). Characterisation of phenolic acid antimicrobial and antioxidant structure-property relationships. *Pharmaceutics*, 12(5), Article 419. <https://doi.org/10.3390/pharmaceutics12050419>
- Lobiuc, A., Pavăl, N. E., Mangalagiu, I. I., Gheorghîță, R., Teliban, G. C., Amăriucăi-Mantu, D., & Stoleru, V. (2023). Future antimicrobials: natural and functionalized phenolics. *Molecules*, 28(3), Article 1114. <https://doi.org/10.3390/molecules28031114>

- Mamdouh, D., & Smetanska, I. (2022). Optimisation of callus and cell suspension cultures of *Lycium schweinfurthii* for improved production of phenolics, flavonoids, and antioxidant activity. *Horticulturae*, 8(5), Article 394. <https://doi.org/10.3390/horticulturae8050394>
- Mathur, M. (2018). Effect of fungal elicitation on growth and metabolite production in callus of few medicinal plants. *International Journal of Life Sciences, Special Issue A9*, 114-116. <https://oaji.net/articles/2017/736-1518498746.pdf>
- Mehaboob, V. M., Faizal, K., Shamsudheen, K. M., Raja, P., Thiagu, G., & Shajahan, A. (2019). direct organogenesis and microrrhizome production in ginger (*Zingiber officinale* Rosc.). *Journal of Pharmacognosy and Phytochemistry*, 8(3), 2880-2883. <https://www.phytojournal.com/archives/2019/vol8issue3/PartAO/8-2-553-417.pdf>
- Miri, S. M. (2020). Micropropagation, callus induction and regeneration of ginger (*Zingiber officinale* Rosc.). *Open Agriculture*, 5(1), 75-84. <https://doi.org/10.1515/opag-2020-0008>
- Nabi, N., Singh, S., & Saffeullah, P. (2021). Responses of *in vitro* cell cultures to elicitation: Regulatory role of jasmonic acid and methyl jasmonate: A review. *In Vitro Cellular & Developmental Biology - Plant*, 57, 341-355. <https://doi.org/10.1007/s11627-020-10140-6>
- Narayani, M., & Srivastava, S. (2017). Elicitation: A stimulation of stress in *in vitro* plant cell/tissue cultures for enhancement of secondary metabolite production. *Phytochemistry Reviews*, 16(6), 1227-1252. <https://doi.org/10.1007/s11101-017-9534-0>
- Nataraj, N., Hussain, M., Ibrahim, M., Hausmann, A. E., Rao, S., Kaur, S., Khazir, J., Mir, B. A., & Olsson, S. B. (2022). Effect of altitude on volatile organic and phenolic compounds of *Artemisia brevifolia* Wall ex DC. from the Western Himalayas. *Frontiers in Ecology and Evolution*, 10, Article 864728. <https://doi.org/10.3389/fevo.2022.864728>
- Nurdyansyah, F., & Widyastuti, D. A. (2022). *Jahe Merah* [Red ginger]. Penerbit Widina.
- Padilha da Silva, W., Lopes, G. V., Ramires, T., & Kleinubing, N. R. (2024). May phenolics mitigate the antimicrobial resistance in foodborne pathogens? *Current Opinion in Food Science*, 55, Article 101107. <https://doi.org/10.1016/j.cofs.2023.101107>
- Phillips, G. C., & Garda, M. (2019). Plant tissue culture media and practices: an overview. *In Vitro Cellular and Developmental Biology - Plant*, 55(3), 242-257. <https://doi.org/10.1007/s11627-019-09983-5>
- Pradhan, B., Patra, S., Dash, S. R., Nayak, R., Behera, C., & Jena, M. (2021). Evaluation of the anti-bacterial activity of methanolic extract of *Chlorella vulgaris* Beyerinck [Beijerinck] with special reference to antioxidant modulation. *Future Journal of Pharmaceutical Sciences*, 7, Article 17. <https://doi.org/10.1186/s43094-020-00172-5>
- Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R. M., & Palazon, J. (2016). Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules*, 21(2), Article 182. <https://doi.org/10.3390/molecules21020182>
- Rani, M., Shylaja, M. R., Mathew, D., Girija, D., Shankar, M. A., Sureshkumar, P., & Beena, C. (2022). Potential of microrrhizomes for *in vitro* gingerol and shogaol synthesis in ginger (*Zingiber officinale* Rosc.). *Proceedings of the National Academy of Sciences India Section B - Biological Sciences*, 92(1), 121-129. <https://doi.org/10.1007/s40011-021-01314-2>

- Sagharyan, M., Ganjeali, A., Cheniany, M., & Kouhi, S. M. M. (2020). Optimisation of callus induction with enhancing production of phenolic compounds production and antioxidants activity in callus cultures of *Nepeta binaloudensis* Jamzad (Lamiaceae). *Iranian Journal of Biotechnology*, 18(4), 47-55. <https://doi.org/10.30498/IJB.2020.2621>
- SeleneMárquez-Rodríguez, A., Nevárez-Baca, S., Lerma-Hernández, J. C., Hernández-Ochoa, L. R., Nevárez-Moorillon, G. V., Gutiérrez-Méndez, N., Muñoz-Castellanos, L. N., & Salas, E. (2020). In vitro antibacterial activity of *Hibiscus sabdariffa* L. phenolic extract and its in situ application on shelf-life of beef meat. *Foods*, 9(8), Article 1080. <https://doi.org/10.3390/foods9081080>
- Suciylati, S. W., & Adnyana, I. K. (2017). Red ginger (*Zingiber officinale* Roscoe var *rubrum*): A review. *Pharmacologyonline*, 2, 60-65. https://pharmacologyonline.silae.it/files/archives/2017/vol2/PhOL_2017_2_A006_Suciylati_60_65.pdf
- Widayat, Cahyono, B., Satriadi, H., & Munfarida, S. (2018). Antioxidant activity and total phenolic content in red ginger (*Zingiber officinale*) based drinks. *IOP Conference Series: Earth and Environmental Science*, 102, Article 012025. <https://doi.org/10.1088/1755-1315/102/1/012025>
- Widyastuti, D. A., Fatimah, S., Nuringtyas, T. R., Santosa, D., & Rohman, A. (2025). Total phenolic and flavonoid contents and antioxidant activity of red ginger (*Zingiber officinale* var. *rubrum*) rhizomes from seven different regions in the island of Java, Indonesia. *Food Research*, 9(6), 79-86. [https://doi.org/10.26656/fr.2017.9\(6\).296](https://doi.org/10.26656/fr.2017.9(6).296)
- Widyastuti, D. A., Nuringtyas, T. R., Rohman, A., & Santosa, D. (2026). Altitude-dependent variation in antibacterial properties of red ginger (*Zingiber officinale* var. *rubrum*): Implications for natural anti-*Salmonella* agents. *Advance Sustainable Science, Engineering and Technology*, 8(2), Article 02602012. <https://doi.org/10.26877/asset.v8i2.2781>
- Widyastuti, D. A., Nuringtyas, T. R., Santosa, D., & Rohman, A. (2024a). A perspective on the in vitro tissue culture of red ginger and its impact on antioxidant activity. *Food Research*, 8(4), 193-199. [https://doi.org/10.26656/fr.2017.8\(4\).324](https://doi.org/10.26656/fr.2017.8(4).324)
- Widyastuti, D. A., Santosa, D., Nuringtyas, T. R., & Rohman, A. (2024b). Callus induction of red ginger (*Zingiber officinale* var. *rubrum*) with 2,4-D and Kinetin combination to enhance total phenolic and flavonoid content. *IOP Conference Series: Earth and Environmental Science*, 1364, Article 012051. <https://doi.org/10.1088/1755-1315/1364/1/012051>
- Winson, K. W. S., Chew, B. L., Sathasivam, K., & Subramaniam, S. (2021). Effect of amino acid supplementation, elicitation and LEDs on *Hylocereus costaricensis* callus culture for the enhancement of betalain pigments. *Scientia Horticulturae*, 289, Article 110459. <https://doi.org/10.1016/j.scienta.2021.110459>
- Yudthavorasit, S., Wongravee, K., & Leepipatpiboon, N. (2014). Characteristic fingerprint based on gingerol derivative analysis for discrimination of ginger (*Zingiber officinale*) according to geographical origin using HPLC-DAD combined with chemometrics. *Food Chemistry*, 158, 101-111. <https://doi.org/10.1016/j.foodchem.2014.02.086>
- Zhang, S., Kou, X., Zhao, H., Mak, K. K., Balijepalli, M. K., & Pichika, M. R. (2022). *Zingiber officinale* var. *rubrum*: Red ginger's medicinal uses. *Molecules*, 27(3), Article 775. <https://doi.org/10.3390/molecules27030775>

- Zhao, M., Bai, J., Bu, X., Tang, Y., Han, W., Li, D., Wang, L., Yang, Y., & Xu, Y. (2021). Microwave-assisted aqueous two-phase extraction of phenolic compounds from *Ribes nigrum* L. and its antibacterial effect on foodborne pathogens. *Food Control*, 119, Article 107449. <https://doi.org/10.1016/j.foodcont.2020.107449>
- Złotek, U., Szymanowska, U., Pecio, U., Kozachok, S., & Jakubczyk, A. (2019). Antioxidative and potentially anti-inflammatory activity of phenolics from lovage leaves *Levisticum officinale* Koch elicited with jasmonic acid and yeast extract. *Molecules*, 24(7), Article 1441. <https://doi.org/10.3390/molecules24071441>

Effects of Post-Harvest Environmental Conditions on GABA Accumulation and Physiological Responses in Tea Shoots

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ABSTRACT

γ -Aminobutyric acid (GABA) is a functional metabolite associated with tea quality and potential health benefits, and it is known to accumulate in plants under abiotic stress conditions. However, information regarding the physiological response and GABA accumulation in harvested tea shoots during post-harvest handling remains limited. Therefore, this study evaluates the effects of several post-harvest environmental conditions, including water status, temperature, light availability, and treatment duration, on GABA accumulation and the physiological response of harvested tea shoots under controlled conditions. The research was conducted in 2022 using tea shoots of the TTTRI-12 cultivar consisting of one bud and three leaves. The shoots were harvested from the experimental garden of National Chung Hsing University and arranged in a completely randomised design with three replicates. The results showed that various post-harvest stress treatments significantly affected GABA content, proline levels, water content, and stomatal conductance. An overall increase in GABA content was observed in treatments involving soaking, high temperature (37.5°C), dark conditions, and a 2-hour treatment. Pearson correlation analysis indicated that proline was the only physiological parameter significantly associated with GABA accumulation. These findings suggest

that harvested tea shoots maintain physiological activity and metabolic activity during the post-harvest period. Overall, manipulating post-harvest environmental conditions has the potential to support the development of GABA tea and improve its quality.

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INTRODUCTION

The tea plant (*Camellia sinensis* L.) is a globally favoured beverage, recognised for its diverse bioactive constituents that enhance its quality and health benefits (Khan & Mukhtar, 2013, 2019). In addition to catechins and amino acids, gamma-aminobutyric acid (GABA) has become an important functional component in the development of tea products. GABA is a non-proteinogenic amino acid that accumulates in plant tissues due to numerous abiotic stressors (Kaspal et al., 2021; Wu et al., 2018; Yong et al., 2017). In tea products, elevated GABA levels correlate with functional value and potential health benefits, including mitigation of insomnia, depression, and anxiety, thereby reducing stress-induced increases in blood pressure (Lin et al., 2023). These beneficial effects are driving continuous initiatives to increase GABA levels in tea leaves.

Tea leaves accumulate GABA in response to abiotic stress. Previous research on diverse species of intact plants has shown that GABA enhances stress tolerance during chilling, drought, low-light, and heat stress by maintaining elevated relative water content (RWC), facilitating osmotic adjustment, stimulating proline and sugar accumulation, improving photosynthetic efficiency and stomatal conductance, and activating antioxidant defense mechanisms while reducing oxidative stress indicators (Liang et al., 2026; Malekzadeh et al., 2014; Nayyar et al., 2014; Yong et al., 2017). GABA accumulation in harvested tea plants, especially in oxygen-deficient conditions, has been studied. Post-harvest anaerobic conditions can increase tea leaf GABA concentration by eightfold, exceeding 1.5 mg/g after multiple aerobic and anaerobic incubations (Tsushida & Murai, 1987; Wu et al., 2018). The studies indicate that postharvest environmental factors significantly affect GABA accumulation in tea leaves.

In plants, GABA accumulation is associated with proline accumulation in response to several abiotic stressors. As glutamate metabolites, proline and GABA are linked to plant stress responses. Glutamate can be converted into GABA by glutamate decarboxylase (GAD) or proline by pyrroline-5-carboxylate (P5C) (Y. Wang et al., 2020). Proline accumulation can remain after rice leaves are detached from the parental plant, suggesting metabolic activity in post-harvest leaves (Chen et al., 2001). This was also shown in hypoxic post-harvest tea leaves (Siahaan & Palupi, 2025). These conditions indicate that several physiological changes may still occur during harvesting, postharvest, and before tea processing.

Post-harvest leaf physiology affects stomatal behaviour. Physiological indicators such as stomatal conductance are affected by environmental factors including light, water availability, temperature, CO₂ concentration, and abiotic stress (Faralli et al., 2019; Xu & Zhou, 2008). In general, harvested leaves degrade slowly rather than immediately reaching minimum stomatal conductance (Duursma et al., 2018). Given that stomata regulate gas exchange, including oxygen diffusion, changes in stomatal conductance may influence GABA accumulation during post-harvest stress (Lawson & Blatt, 2014; X. Mei et al., 2016).

Previous studies have extensively discussed the role and accumulation of GABA in various plant species experiencing abiotic stress (Liang et al., 2026; Malekzadeh et al., 2014; Nayyar et al., 2014; Yong et al., 2017). Additionally, increased GABA accumulation in tea leaves due to anaerobic treatment and its correlation with various plant physiological responses have been reported (Liao et al., 2017; Tsushida & Murai, 1987; Wu et al., 2018). However, Research on GABA accumulation and physiological responses of tea shoots under post-harvest abiotic stress remains limited, despite the known influence of pre- and post-harvest stress on metabolites related to tea quality (Shao et al., 2021).

Water status affects tissue hydration (da S. Lopes et al., 2024); temperature affects cellular metabolism (Marchin et al., 2022; Priya et al., 2019); light exposure during tea shoot harvesting and storage can alter stomatal regulation and leaf physiological activity (Ye et al., 2021); and stress duration affects metabolic responses. These changes may trigger physiological and biochemical responses associated with GABA accumulation in tea shoots during post-harvest processing. This study examines how post-harvest environmental conditions, including water status, temperature, light availability, and treatment duration, affect GABA, proline accumulation, water content, and stomatal conductance in tea shoots, as well as the correlations among these parameters. Field conditions during tea harvesting determined treatment considerations. The results of this research are expected to contribute to a deeper understanding of post-harvest stress physiology in tea shoots and provide useful information for the development of GABA tea processing.

MATERIALS AND METHODS

This experiment was conducted from May to December 2022 at the Agronomy Department Lab of National Chung Hsing University (NCHU) in Taiwan. The tea leaves cultivar TTRI-12 were used as the sample for this experiment and were obtained from the NCHU practice field. Although the ages of individual plants in the field varied, the sample selection was based on uniform tip maturity and consistent harvesting standards to minimise physiological variation among samples. The harvesting criteria used for the samples in this study consisted of one bud and three leaves to mimic the actual condition of tea leaves typically used in a tea beverage product (Aaqil et al., 2023).

Experimental Design and Sample Preparation

The experiment was conducted as a factorial experiment arranged in a completely randomised design (CRD). The treatment factors were leaf water status (soaking and withering); treatment duration (2, 4, and 8 hours); treatment temperature (25 °C and 37.5 °C); and light conditions (light at $94 \mu\text{mol m}^{-2} \text{s}^{-1}$ and dark). The initial sample was used as the control before treatment and served as a baseline for evaluating physiological changes after treatment application. After harvesting, fresh tea shoots were divided into three groups: control, soaking, and withering. In the soaking treatment, tea shoot stems

were soaked in water to maintain freshness and turgidity during storage, whereas in the withering treatment, they were only placed in trays. Both soaking and withering treatments were applied under combinations of two temperatures (25 °C and 37.5 °C), two light conditions (light and dark), and three treatment durations (2, 4, and 8 hours). At the end of each treatment duration, samples were immediately stored at -20 °C until further simultaneous physiological analysis. Storage at -20 °C for both the control and other treatments was intended to ensure that all treatments were analysed together under the same conditions, thereby minimising inter-analysis variation and maintaining consistency among measurements. The sample size per treatment was 4-5 tea shoots, and the treatment was conducted in a growth chamber with 3 replicates.

Determination of Water Content

For water content determination, the samples were ground in liquid nitrogen and stored at -20 °C until analysis. Approximately 0.3-0.5 g of the sample mass was transferred into an Eppendorf tube and dried in an oven at 70 °C for 48 hours.

Determination of Stomatal Conductance

Stomatal conductance was measured with a leaf porometer (SC-1, METER Group, Inc., Pullman, WA, USA) on the abaxial surface of the tea leaf. For the control group, measurements were taken immediately after harvest; for the tea groups exposed to water status, duration, temperature, and light conditions, checks were conducted immediately after treatment completion.

Determination of Proline Content

For proline determination, samples were ground with liquid nitrogen, placed into tubes, and stored at -20 °C. After all the samples were ground, between 0.3-0.5 g of each sample was placed in an Eppendorf tube. Proline analysis was based on the procedure from Bates et al. (1973) with some modifications. A total of 1.5 mL of Sulfosalicylic acid 3% (w/v) was added to the sample, which was then centrifuged for 20 minutes at 25 °C and 10,000 g. For the analysis, a total of 0.5 mL of the supernatant was used for the proline assay. The ninhydrin reagent was prepared by dissolving 0.25 g of ninhydrin in a mixture of 6 mL of acetic acid and 4 mL of 6 M H₃PO₄. A total of 0.5 mL of filtrate was reacted with 0.5 mL of ninhydrin acid solution and 0.5 mL of acetic acid, then incubated for 1 hour at 100 °C, and finally immersed in an ice bath to stop the reaction. Then the mixture was extracted with 2 mL of toluene and vigorously shaken in a vortex mixer for 15-20 seconds. The absorbance was measured at 520 nm with the spectrophotometer in cuvette mode (SpectraMax M2, Molecular Devices, San Jose, CA, USA). Toluene was used for the blank. A standard curve was generated using known concentrations of pure proline for quantification.

Determination of GABA Content

For GABA determination, samples were ground in liquid nitrogen, placed in tubes, and stored at 20 °C. After all the samples were ground, 0.3-0.5 g of each sample was placed in an Eppendorf tube. For the GABA extraction, the tea leaf samples were mixed with 1 mL of hot water and allowed to stand for 6 minutes. A 250 µl reagent solution containing 300 mL of o-phthalaldehyde (OPA) stock solution, 20 µl of 3-mercaptopropionic acid, and 4 mL of borate buffer was added to a 50 µl aliquot of the supernatant. The mixture was then filtered using a syringe filter, and 20 µl was injected into an HPLC (High-Performance Liquid Chromatography) system (Model LC-10AD VP, Shimadzu, Japan). For the HPLC analysis, mobile phase I consisted of 90 mL of phosphate buffer mixed with 370 mL of ddH₂O, and mobile phase II consisted of acetonitrile (HPLC grade). The stock solution of OPA was prepared by dissolving 100 mg OPA in 3 mL of methanol. The borate buffer was prepared by dissolving 4.77 g of boric acid in approximately 475 mL of water. The pH of the buffer was adjusted to 10.0 using 12 M and 1 M NaOH, and the final volume was also adjusted to 500 ml with ddH₂O. The phosphate buffer was prepared by dissolving 3.54 g KH₂PO₄ and 5.66 g Na₂HPO₄ in 800 mL of distilled water, adjusting the pH to 7.0 with 50% (w/v) NaOH, and making the final volume up to 1 L. The HPLC column was SHARPSIL C18 (4.6 mm × 25 cm, 5 µm), and a UV-Vis detector was used. Gradient elution profile: 0-15 minutes was kept at 89% of buffer A; 15-17 minutes, linear gradient change to 50% of buffer A; 17-20 minutes, kept at 50% of buffer A; 20-22 minutes, linear gradient change to 89% of buffer A; 22-30 minutes, kept at 89% of buffer A. 0.8 ml/minute was set as the flow rate, and spectrofluorometric detection was at an absorbance wavelength of 228 nm.

Data Analysis

The data were analysed using the General Linear Model (GLM) procedure to assess the main effects of water status, temperature, light conditions, and treatment duration on GABA accumulation and physiological responses in tea shoots. Mean comparisons among treatment levels were conducted using the Least Significant Difference (LSD) test at $P < 0.05$. Correlation analysis was used to determine relationships between parameters. Pearson correlation analysis was performed using pooled data from all treatment combinations.

RESULT

Effect of Water Status on GABA Accumulation and Physiological Responses

The various water status levels affected GABA, proline, water content, and stomatal conductance ($p < 0.05$) (Figure 1). The water treatment factor significantly influenced GABA accumulation. Soaking treatment resulted in the highest GABA concentration, at 0.09 mg/g DW, which was significantly higher than the control (0.05 mg/g DW). Meanwhile, the

withering treatment showed an intermediate GABA concentration of 0.07 mg/g DW, but it was not significantly different from the soaking treatment and the control (Figure 1a).

A different pattern from GABA was found in proline accumulation. The withering treatment resulted in the highest proline concentration, at 10.72 $\mu\text{mol/g DW}$, although it was not significantly different from that of the soaking treatment (9.02 $\mu\text{mol/g DW}$). Meanwhile, the control treatment accumulated the least proline, at 5.28 $\mu\text{mol/g DW}$, which was significantly lower than the soaking and withering treatments (Figure b).

The soaking treatment maintains the highest water content at 73.95%, with no significant difference from the control at 71.77%. Conversely, the withering treatment significantly reduces the water content to 65.23%, indicating water loss (Figure 1c). Similarly, stomatal conductance decreases significantly in each water-status treatment. The tea shoot in the control condition shows the highest stomatal conductance, approximately 29.03 $\text{mmol/m}^2/\text{s}$, which then decreases in the soaking treatment to 19.47 $\text{mmol/m}^2/\text{s}$, and reaches the lowest value in the withering treatment at 3.51 $\text{mmol/m}^2/\text{s}$ (Figure 1d).

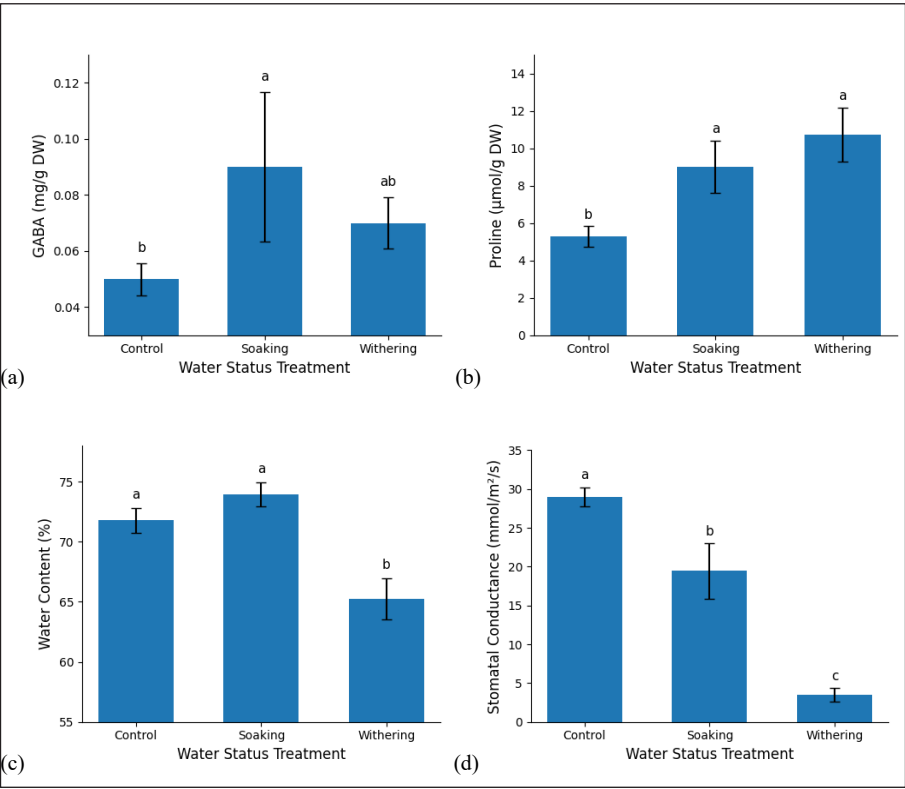


Figure 1. The GABA (a), proline (b), water content (c), and stomatal conductance (d) change in water status treatment. Statistical analysis using least significant difference (LSD). Bars with different letters indicate significant differences ($P < 0.05$)

Effect of temperature on GABA accumulation and physiological responses

Treatment of abiotic stress caused by temperature differences results in the highest GABA accumulation at 37.5 °C. The GABA concentration accumulated higher when placing tea shoots at 37.5 °C (0.11 mg/g DW), which is significantly different from the control treatment (0.05 mg/g DW) and at 25 °C (0.06 mg/g DW). This indicates that the lowest GABA accumulation occurs in the control treatment and that it gradually increases with rising storage temperature (Figure 2a).

The highest proline accumulation was observed when the storage temperature of the tea shoots was increased to 37.5 °C (12.65 µmol/g DW), which was significantly higher than the control treatment and the 25 °C treatment. The 25 °C temperature treatment also tended to increase proline accumulation to 7 µmol/g DW compared with the control (5 µmol/g DW), although the difference was not significant (Figure 2b).

Water content of tea shoots decreased significantly at 37.5 °C, reaching a minimum of 67%, which differed significantly from the control and 25 °C treatments.

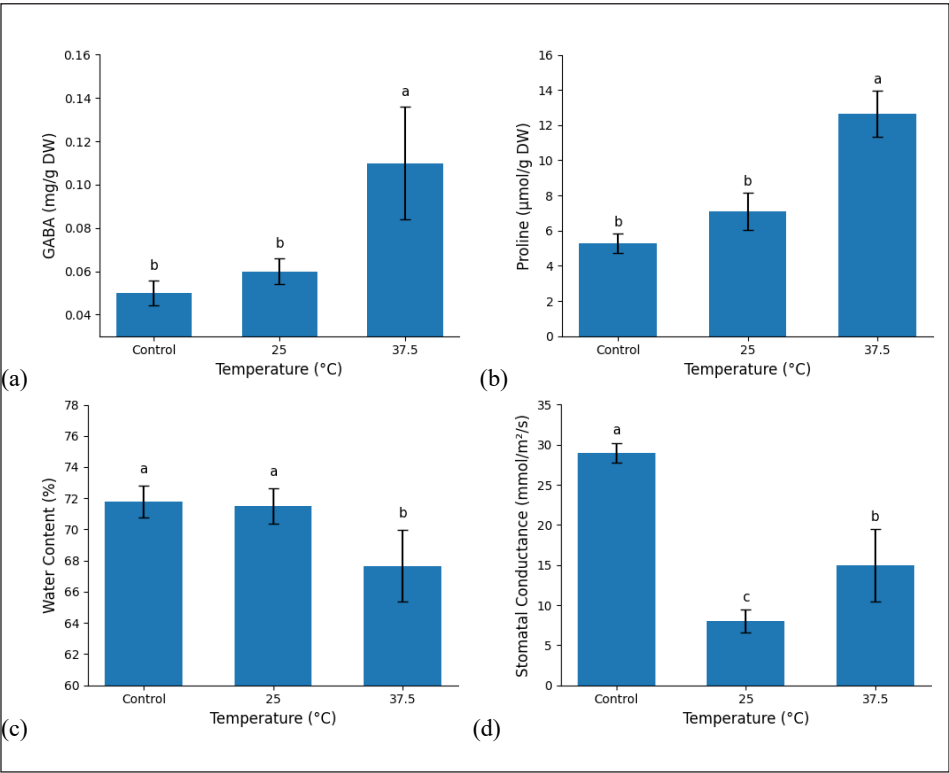


Figure 2. The GABA (a), proline (b), water content (c), and stomatal conductance (d) change with temperature treatment. Statistical analysis using least significant difference (LSD). Bars with different letters indicate significant differences ($P<0.05$) ($n = 3$)

Meanwhile, at 25 °C, there was no significant difference in the water content of tea shoots compared with the control treatment. In the control treatment, the water content of the tea shoots was 71% and decreased slightly to 70% when the storage temperature was increased to 25 °C (Figure 2c). However, for stomatal conductance, the highest value is observed in the control treatment (29.04 mmol/m²/s), then decreases significantly and reaches its lowest value at 25 °C (8.03 mmol/m²/s). Furthermore, as the temperature increases to 37.5 °C, stomatal conductance rises to 14.95 mmol/m²/s and differs significantly from the 25 °C treatment (Figure 2d).

Effect of Light Availability on GABA Accumulation and Physiological Responses

Light availability treatment had a significant effect on changes in GABA accumulation in tea shoots (Figure 3a). Tea shoots that received dark treatment after harvesting appeared to accumulate the highest GABA, at 0.09 mg/g DW, which was significantly higher than the control treatment (0.05 mg/g DW) but not significantly different from the light treatment (0.07 mg/g DW). Conversely, light and dark treatments showed a significantly greater increase in proline accumulation than the control treatment (Figure 3b). The proline concentration accumulated in the light treatment was 9.90 µmol/g DW, which was not significantly different from that in the dark treatment (9.84 µmol/g DW). The initial or control proline concentration was 5.28 µmol/g DW.

Under light and dark conditions, water content in tea shoot tissue decreased from 71.77% in the control treatment to 70.48% in the light treatment. This decrease appears very small and is not statistically significant. Unlike the dark treatment, the water content decrease was very low (68.69%) and significantly different from the control treatment, but not significantly different from the light treatment (Figure 3c).

Among all the physiological parameters observed, stomatal conductance showed the most significant response to the light availability treatment (Figure 3d). The highest stomatal conductance was observed in the control condition (29.03 mmol m⁻² s⁻¹), which was significantly higher than in the light and dark treatments. The light treatment resulted in a stomatal conductance of 14.34 mmol m⁻² s⁻¹, while the lowest value was found in the dark treatment, at 8.64 mmol m⁻² s⁻¹.

Effect of Treatment Duration on GABA Accumulation and Physiological Responses

The treatment duration of abiotic stress showed a significant effect on GABA accumulation in tea shoots. The control treatment (0 h) showed the lowest GABA accumulation, at 0.05 mg/g DW. As storage time increased to 2 hours, GABA accumulation increased significantly compared to the control treatment and reached the highest level, at 0.10 mg/g DW. However, the difference was not statistically significant compared with

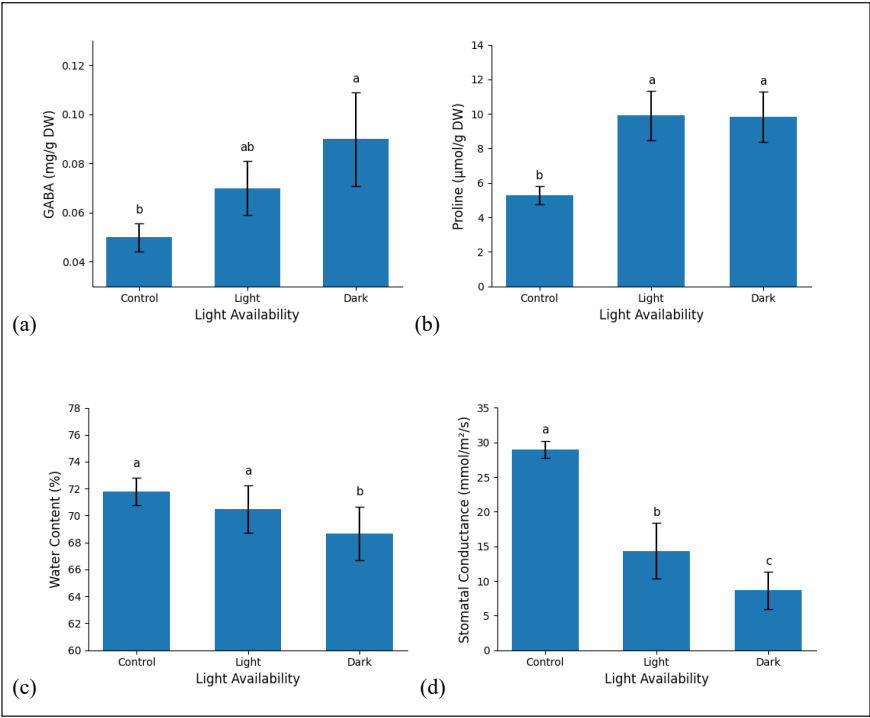


Figure 3. The GABA (a), proline (b), water content (c), and stomatal conductance (d) change in light availability treatment. Statistical analysis using least significant difference (LSD). Bars with different letters indicate significant differences ($P < 0.05$) ($n = 3$)

tea shoots treated for 4 hours (0.07 mg/g DW) and 8 hours (0.08 mg/g DW). This trend indicates that GABA content gradually declined after peaking at 2 hours (Figure 4a).

The same trend was observed in proline accumulation resulting from treatments with different durations of abiotic stress (Figure 4b). In the control conditions, proline concentration was the lowest among treatments with increased durations, at 5.28 μmol/g DW. Proline accumulation reached its peak at 2 hours, at 9.46 μmol/g DW, which was not significantly different from the treatments at 4 hours (8.70 μmol/g DW) and 8 hours (11.46 μmol/g DW). However, generally, the pattern of accumulation change indicates that proline accumulation increased during the 2-hour treatment, then decreased at 4 hours, and gradually increased significantly at 8 hours.

The variation in treatment duration also significantly affects changes in water content in tea shoot tissue (Figure 4c). Control treatment showed a tissue water content of 71.77%, which then gradually decreased during the 2-hour treatment (71.72%), although this decrease was not significant. As the duration of the treatment increased, the water content continued to decrease significantly at 4 hours (69.43%) and 8 hours (68.05%) compared to the control condition.

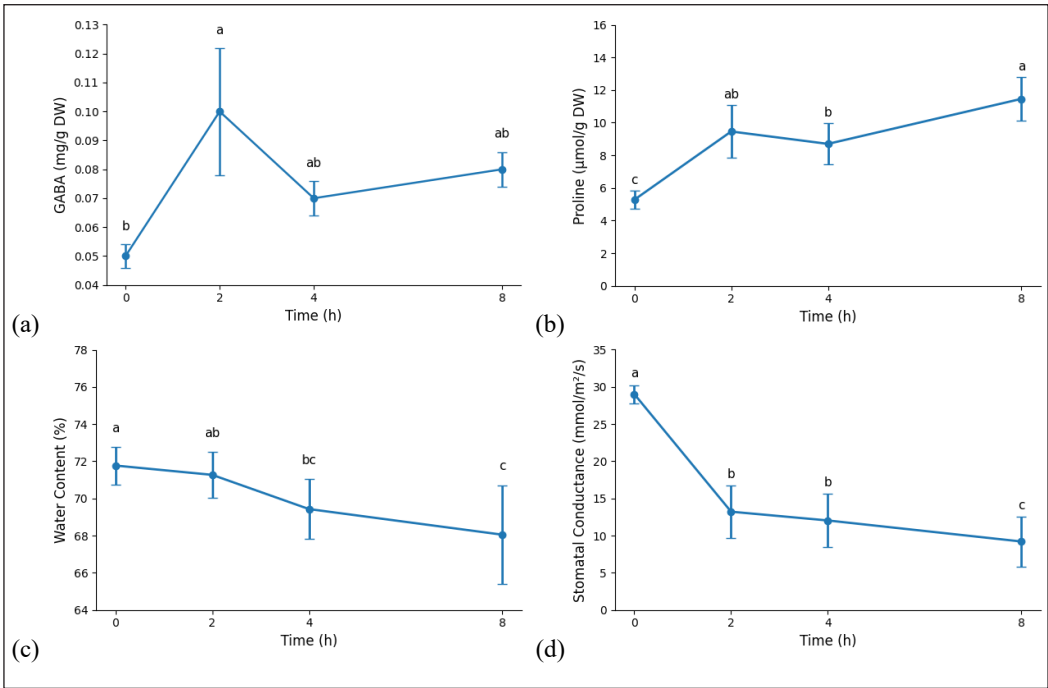


Figure 4. The GABA (a), proline (b), water content (c), and stomatal conductance (d) change in different treatment durations. Statistical analysis using least significant difference (LSD). Dots with different letters indicate significant differences ($P<0.05$) ($n = 3$)

Unlike water content, the initial shoot condition (control treatment) had the highest stomatal conductance (29.03 mmol/m²/s), which then decreased significantly with increasing treatment duration. However, stomatal conductance at the 2-hour treatment (13.22 mmol/m²/s) was not significantly different from that at the 4-hour treatment (12.04 mmol/m²/s), but it was significantly different from that at the 8-hour treatment (9.21 mmol/m²/s) (Figure 4d).

Pearson Correlation among GABA and Physiological Parameters

Pearson correlation analysis indicates varying relationships between GABA accumulation and physiological parameters of tea shoots during post-harvest stress treatment (Table 1). GABA shows a highly significant positive correlation with proline, but there is no significant correlation with other physiological responses, namely water content and stomatal conductance. Conversely, proline shows a significant negative correlation with water content and stomatal conductance. Meanwhile, for the water content and stomatal conductance parameters, a highly significant positive correlation was also observed.

Table 1
Correlation between GABA, proline, water content, and stomata conductance in tea shoots exposed to several water statuses, temperature, light availability, and treatment duration

Parameter	Water Content	Stomatal Conductance	Proline
GABA	0.146 ns	0.127 ns	0.200**
Water Content		0.468**	-0.266**
Stomatal Conductance			-0.192*

Note. Asterisks * and ** denote statistical significance at $P \leq 0.05$ and $P \leq 0.01$, respectively. ns, not significant. All the numbers are obtained from the Pearson correlation

DISCUSSION

Post-harvest abiotic stress treatment affects GABA accumulation and physiological responses in tea shoots. In general, stress circumstances such as water status, temperature, light availability, and treatment duration increase GABA content relative to controls. These modifications also affect proline accumulation, water content, and stomatal conductance. This study found that harvested tea shoots retain physiological activity and metabolic processes, enabling them to adapt to environmental changes after harvest. Previous research has shown that post-harvest tea shoots can still respond to environmental stress through physiological and metabolic changes (Shao et al., 2021).

Tea shoots accumulate more GABA after soaking as a response to changes in water status and oxygen availability. Reduced oxygen availability in tea shoot tissues during soaking may increase glutamate decarboxylase (GAD), which turns glutamate into GABA via the GABA shunt pathway (Crawford et al., 1994; Liao et al., 2017). In addition to water-status treatment, high temperature (37.5 °C) also promotes GABA accumulation. GABA accumulation in intact plants is associated with membrane protection and mitochondrial activity under heat stress (Nayyar et al., 2014; Priya et al., 2019), which may explain the increased GABA content in this study under high temperatures.

Dark conditions tend to increase GABA accumulation compared with light conditions. In detached tea shoots, the absence of light temporarily halts photosynthesis, while cellular respiration continues, leading to CO₂ accumulation and a decrease in intracellular pH. This acidic condition activates the enzyme GAD, which converts glutamate into GABA via the GABA shunt pathway (Yogeswara et al., 2020). Furthermore, during treatment, GABA content tends to increase with prolonged postharvest stress, suggesting that prolonged stress may intensify metabolic changes in tea shoots.

Proline plays a role in maintaining osmotic stability and protecting plant cells during abiotic stress. The withering and soaking treatments increase proline levels relative to the control because both trigger post-harvest stress: withering induces osmotic stress from water loss (Lv et al., 2011), while soaking induces hypoxia due to low oxygen availability

(Siahaan & Palupi, 2025). Although proline levels during withering tend to be higher than during soaking, the difference is not significant, indicating that both treatments induce a similar level of physiological stress that triggers proline accumulation.

Treatment at 37.5 °C significantly increased proline accumulation compared to the control and 25.5 °C, indicating the role of proline as a reactive oxygen species (ROS) scavenger and protein stabiliser during heat stress in tea shoots (Roychoudhury et al., 2015; Xiong et al., 2012). Under light availability treatment, both light and dark conditions differed significantly from the control, but did not differ significantly from each other. Proline content in dark conditions tended to be lower than in light, which is suspected to be related to the use of carbohydrate reserves during post-harvest respiration, considering that proline synthesis is influenced by carbohydrate availability (Stewart, 1972). Meanwhile, the highest proline levels were observed at an 8-hour treatment duration, although they were not significantly different from those at the 2-hour treatment. Based on previous research, this increase in proline is associated with a sharp spike in Pyrroline-5-carboxylate synthetase gene expression during the first 2 hours of stress (H. Wang et al., 2015).

Water content and stomatal conductance show consistent physiological changes in response to various post-harvest stress treatments on tea shoots. The withering treatment causes a significantly greater decrease in water content than the control or soaking, indicating water loss due to the withering process. Conversely, the soaking treatment maintains a higher water content due to direct contact between the tissue and water (Zeng et al., 2024). In terms of temperature treatment, exposure to 37.5 °C increases water loss compared to the control and 25 °C, although not significantly, whereas dark conditions and longer treatment durations (4 and 8 hours) significantly reduce water content. The decrease in water content during the treatment is suspected to be related to increased post-harvest respiration and transpiration, which leads to greater utilisation of tissue water reserves as the treatment duration lengthens (S. Mei et al., 2022).

The change in water content is in line with the response of stomatal conductance, which tends to decrease under stress conditions. Soaking treatment reduces stomatal conductance compared to the control and experiences a sharper decline during withering. This finding further indicates that as leaves lose water content, stomatal conductance correspondingly decreases. Stomatal conductance depends on the plant's water potential or hydration status (Giménez et al., 2013). In temperature treatments, the lowest stomatal conductance was observed at 25 °C and was significantly different from both the control and 37.5 °C. This decrease is likely due to stomatal closure from water stress after tea shoots are harvested. However, the increase in stomatal conductance again at 37.5 °C may be due to disrupted stomatal regulation under heat stress, leading to passive stomatal leakage even when the tissue is under stress (Marchin et al., 2022). Light treatment results in higher stomatal conductance compared to darkness. Light directly or indirectly influences the biosynthesis

and degradation of Absciscic acid (ABA) (Ye et al., 2021). Degradation of ABA may occur, resulting in continued stomatal opening due to the absence of closure signals. Absciscic acid (ABA) is a plant hormone that responds to abiotic and biotic stresses, significantly affecting stomatal movement and transpiration (Bharath et al., 2021). For the duration treatment, an 8-hour treatment resulted in the largest decrease in stomatal conductance compared with other treatments.

Pearson's correlation indicates that water content is strongly positively correlated with stomatal conductance, meaning that the higher the water content in leaves, the wider the stomatal openings. Conversely, a decrease in water content triggers stomatal closure to prevent dehydration (da S. Lopes et al., 2024). Proline shows a significant negative correlation with water content and stomatal conductance, indicating its role in the plant's natural defence mechanism (osmoregulation) when facing stress (Hayat et al., 2012). Conversely, GABA shows no significant correlation with water content or stomatal conductance but a highly significant positive correlation with proline. This suggests that GABA accumulation is more closely related to metabolic and biochemical responses to post-harvest stress than to direct physiological changes in water status and stomatal activity.

CONCLUSION

This study reveals that post-harvest water status, temperature, light availability, and treatment time affect GABA accumulation and physiological responses in harvested tea shoots. Generally, post-harvest abiotic stress treatments enhance GABA and proline and decrease water content and stomatal conductance compared to the initial condition after picking. Pearson correlation analysis indicates that proline is a physiological parameter affecting GABA accumulation. These findings imply that post-harvest tea shoots still maintain active physiological activity and metabolism, allowing them to respond to environmental changes after harvesting.

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REFERENCE

Aaqil, M., Peng, C., Kamal, A., Nawaz, T., Zhang, F., & Gong, J. (2023). Tea harvesting and processing techniques and its effect on phytochemical profile and final quality of black tea: A review. *Foods*, 12(24), Article 4467. <https://doi.org/10.3390/foods12244467>

- Bharath, P., Gahir, S., & Raghavendra, A. S. (2021). Absciscic acid-induced stomatal closure: An important component of plant defense against abiotic and biotic stress. *Frontiers in Plant Science*, 12, Article 615114. <https://doi.org/10.3389/fpls.2021.615114>
- Chen, C. T., Chen, L.-M., Lin, C., & Kao, C. H. (2001). Regulation of proline accumulation in detached rice leaves exposed to excess copper. *Plant Science*, 160(2), 283-290. [https://doi.org/10.1016/S0168-9452\(00\)00393-9](https://doi.org/10.1016/S0168-9452(00)00393-9)
- Crawford, L. A., Bown, A. W., Breitkreuz, K. E., & Guinel, F. C. (1994). The synthesis of γ -aminobutyric acid in response to treatments reducing cytosolic pH. *Plant Physiology*, 104(3), 865–871. <https://doi.org/10.1104/pp.104.3.865>
- da S. Lopes, A., de Andrade Júnior, A. S., Bastos, E. A., de Sousa, C. A. F., Casari, R. A. D. C. N., & de Moura, M. S. B. (2024). Detecção do estado hídrico em híbridos de milho com imagens aéreas obtidas por aeronave remotamente pilotada [Assessment of maize hybrid water status using aerial images from an unmanned aerial vehicle]. *Revista Caatinga*, 37, Article e11701. <https://doi.org/10.1590/1983-21252024v37i11701rc>
- Duursma, R. A., Blackman, C. J., Lopez, R., Martin-StPaul, N. K., Cochard, H., & Medlyn, B. E. (2018). On the minimum leaf conductance: Its role in models of plant water use, and ecological and environmental controls. *New Phytologist*, 221(2), 693–705. <https://doi.org/10.1111/nph.15395>
- Faralli, M., Matthews, J., & Lawson, T. (2019). Exploiting natural variation and genetic manipulation of stomatal conductance for crop improvement. *Current Opinion in Plant Biology*, 49, 1–7. <https://doi.org/10.1016/j.pbi.2019.01.003>
- Giménez, C., Gallardo, M., & Thompson, R. B. (2013). Plant–water relations. In Daniel Hillel (Ed.), *Reference Module in Earth Systems and Environmental Sciences*, 231-238, Elsevier. <https://doi.org/10.1016/B978-0-12-409548-9.05257-X>
- Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., & Ahmad, A. (2012). Role of proline under changing environments: A review. *Plant Signaling & Behavior*, 7(11), 1456–1466. <https://doi.org/10.4161/psb.21949>
- Kaspal, M., Kanapaddalagamage, M. H., & Ramesh, S. A. (2021). Emerging roles of γ -aminobutyric acid (GABA) gated channels in plant stress tolerance. *Plants*, 10(10), Article 2178. <https://doi.org/10.3390/plants10102178>
- Khan, N., & Mukhtar, H. (2013). Tea and health: Studies in humans. *Current pharmaceutical design*, 19(34), 6141–6147. <https://doi.org/10.2174/1381612811319340008>
- Khan, N., & Mukhtar, H. (2019). Tea polyphenols in promotion of human health. *Nutrients*, 11(1), Article 39. <https://doi.org/10.3390/nu11010039>
- Lawson, T., & Blatt, M. R. (2014). Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology*, 164(4), 1556–1570. <https://doi.org/10.1104/pp.114.237107>
- Liang, Z., Gao, Y., Zhang, M., Pu, K., Zhang, L., Ma, Y., Huo, J., Zhang, J., Li, J., & Xie, J. (2026). Foliar application of exogenous gamma-aminobutyric acid alleviates the detrimental effects of combined low-temperature and low-light stress in pepper (*Capsicum annuum* L.). *Plant Physiology and Biochemistry*, 235, Article 111338. <https://doi.org/10.1016/j.plaphy.2026.111338>

- Liao, J., Wu, X., Xing, Z., Li, Q., Duan, Y., Fang, W., & Zhu, X. (2017). γ -Aminobutyric acid (GABA) accumulation in tea (*Camellia sinensis* L.) through the GABA shunt and polyamine degradation pathways under anoxia. *Journal of Agricultural and Food Chemistry*, 65(14), 3013–3018. <https://doi.org/10.1021/acs.jafc.7b00304>
- Lin, C.-C., Hsieh, C.-Y., Chen, L.-F., Chen, Y.-C., Ho, T.-H., Chang, S.-C., & Chang, J.-F. (2023). Versatile effects of GABA oolong tea on improvements in diastolic blood pressure, alpha brain waves, and quality of life. *Foods*, 12(22), Article 4101. <https://doi.org/10.3390/foods12224101>
- Lv, W. -T., Lin, B., Zhang, M., & Hua, X. -J. (2011). Proline accumulation is inhibitory to *Arabidopsis* seedlings during heat stress. *Plant Physiology*, 156(4), 1921–1933. <https://doi.org/10.1104/pp.111.175810>
- Malekzadeh, P., Khara, J., & Heydari, R. (2014). Alleviating effects of exogenous gamma-aminobutyric acid on tomato seedling under chilling stress. *Physiology and Molecular Biology of Plants*, 20(1), 133–137. <https://doi.org/10.1007/s12298-013-0203-5>
- Marchin, R. M., Backes, D., Ossola, A., Leishman, M. R., Tjoelker, M. G., & Ellsworth, D. S. (2022). Extreme heat increases stomatal conductance and drought-induced mortality risk in vulnerable plant species. *Global Change Biology*, 28(3), 1133–1146. <https://doi.org/10.1111/gcb.15976>
- Mei, S., Yu, Z., Chen, J., Zheng, P., Sun, B., Guo, J., & Liu, S. (2022). The physiology of postharvest tea (*Camellia sinensis*) leaves, according to metabolic phenotypes and gene expression analysis. *Molecules*, 27(5), Article 1708. <https://doi.org/10.3390/molecules27051708>
- Mei, X., Chen, Y., Zhang, L., Fu, X., Wei, Q., Grierson, D., Zhou, Y., Huang, Y., Dong, F., & Yang, Z. (2016). Dual mechanisms regulating glutamate decarboxylases and accumulation of gamma-aminobutyric acid in tea (*Camellia sinensis*) leaves exposed to multiple stresses. *Scientific Reports*, 6, Article 23685. <https://doi.org/10.1038/srep23685>
- Nayyar, H., Kaur, R., Kaur, S., & Singh, R. (2014). γ -Aminobutyric acid (GABA) imparts partial protection from heat stress injury to rice seedlings by improving leaf turgor and upregulating osmoprotectants and antioxidants. *Journal of Plant Growth Regulation*, 33(2), 408–419. <https://doi.org/10.1007/s00344-013-9389-6>
- Priya, M., Sharma, L., Kaur, R., Bindumadhava, H., Nair, R. M., Siddique, K. H. M., & Nayyar, H. (2019). GABA (γ -aminobutyric acid), as a thermo-protectant, to improve the reproductive function of heat-stressed mungbean plants. *Scientific Reports*, 9(1), Article 7788. <https://doi.org/10.1038/s41598-019-44163-w>
- Roychoudhury, A., Banerjee, A., & Lahiri, V. (2015). Metabolic and molecular-genetic regulation of proline signalling and its cross-talk with major effectors mediates abiotic stress tolerance in plants. *Turkish Journal of Botany*, 39(6), 887–910. <https://doi.org/10.3906/bot-1503-27>
- Shao, C., Zhang, C., Lv, Z., & Shen, C. (2021). Pre- and post-harvest exposure to stress influence quality-related metabolites in fresh tea leaves (*Camellia sinensis*). *Scientia Horticulturae*, 281, Article 109984. <https://doi.org/10.1016/j.scienta.2021.109984>
- Siahaan, L., & Palupi, D. (2025). Analisis kadar prolin daun teh pascapanen dalam kondisi hipoksia [Analysis of proline levels in post-harvest tea leaves under hypoxic conditions]. *Agropross: National Conference Proceedings of Agriculture*, 7, 266–272. <https://doi.org/10.25047/agropross.2025.835>

- Stewart, C. R. (1972). Effects of proline and carbohydrates on the metabolism of exogenous proline by excised bean leaves in the dark. *Plant Physiology*, 50(5), 551–555. <https://doi.org/10.1104/pp.50.5.551>
- Tsushida, T., & Murai, T. (1987). Conversion of glutamic acid to γ -aminobutyric acid in tea leaves under anaerobic conditions. *Agricultural and Biological Chemistry*, 51(11), 2865–2871. <https://doi.org/10.1080/00021369.1987.10868498>
- Wang, H., Tang, X., Wang, H., & Shao, H. B. (2015). Proline accumulation and metabolism-related genes expression profiles in *Kosteletzkya virginica* seedlings under salt stress. *Frontiers in Plant Science*, 6, Article 792. <https://doi.org/10.3389/fpls.2015.00792>
- Wang, Y., Xiong, F., Nong, S., Liao, J., Xing, A., Shen, Q., Ma, Y., Fang, W., & Zhu, X. (2020). Effects of nitric oxide on the GABA, polyamines, and proline in tea (*Camellia sinensis*) roots under cold stress. *Scientific Reports*, 10, Article 12240. <https://doi.org/10.1038/s41598-020-69253-y>
- Wu, Q. Y., Ma, S. Z., Zhang, W. W., Yao, K. B., Chen, L., Zhao, F., & Zhuang, Y. Q. (2018). Accumulating pathways of γ -aminobutyric acid during anaerobic and aerobic sequential incubations in fresh tea leaves. *Food Chemistry*, 240, 1081–1086. <https://doi.org/10.1016/j.foodchem.2017.08.004>
- Xiong, J., Zhang, L., Fu, G., Yang, Y., Zhu, C., & Tao, L. (2012). Drought-induced proline accumulation is uninvolved with increased nitric oxide, which alleviates drought stress by decreasing transpiration in rice. *Journal of Plant Research*, 125, 155–164. <https://doi.org/10.1007/s10265-011-0417-y>
- Xu, Z., & Zhou, G. (2008). Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *Journal of Experimental Botany*, 59(12), 3317–3325. <https://doi.org/10.1093/jxb/ern185>
- Ye, J.-H., Lv, Y.-Q., Liu, S.-R., Jin, J., Wang, Y.-F., Wei, C.-L., & Zhao, S.-Q. (2021). Effects of light intensity and spectral composition on the transcriptome profiles of leaves in shade grown tea plants (*Camellia sinensis* L.) and regulatory network of flavonoid biosynthesis. *Molecules*, 26(19), Article 5836. <https://doi.org/10.3390/molecules26195836>
- Yogeswara, I. B. A., Maneerat, S., & Haltrich, D. (2020). Glutamate decarboxylase from lactic acid bacteria—A key enzyme in GABA synthesis. *Microorganisms*, 8(12), Article 1923. <https://doi.org/10.3390/microorganisms8121923>
- Yong, B., Xie, H., Li, Z., Li, Y. P., Zhang, Y., Nie, G., Zhang, X. Q., Ma, X., Huang, L. K., Yan, Y. H., & Peng, Y. (2017). Exogenous application of GABA improves PEG-induced drought tolerance positively associated with GABA-shunt, polyamines, and proline metabolism in white clover. *Frontiers in Physiology*, 8, Article 1107. <https://doi.org/10.3389/fphys.2017.01107>
- Zeng, Z., Han, C., Wang, Q., Yuan, H., Zhang, X., & Li, B. (2024). Analysis of drying characteristic, effective moisture diffusivity and energy, exergy and environment performance indicators during thin layer drying of tea in a convective-hot air dryer. *Frontiers in Sustainable Food Systems*, 8, Article 1371696. <https://doi.org/10.3389/fsufs.2024.1371696>

Review Article

A Critical Review of *Leucaena leucocephala* Leaves as an Alternative Source of Dietary Supplement on the Production Performance of Growing Rabbits

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ABSTRACT

Rabbit meat is a potential option to conventional red meat due to its higher crude protein content (20-25%), lower fat level (6-10%), and rich nutrient profile. Despite these nutritional benefits, rabbit production remains underdeveloped in many regions due to limited market access, inadequate consumer awareness, and high cost of feed, which accounts for nearly 60-70% of total production expenses. Consequently, the development of cost-effective feeding strategies is essential to improve the profitability and sustainability of rabbit farming. One promising approach is the inclusion of alternative and locally available feed resources, such as *Leucaena leucocephala* (LL) leaves. Dietary supplementation with LL leaves at low to moderate levels (up to 10%) can improve growth performance, feed intake, nutrient utilisation, feed conversion efficiency, carcass quality, and economic returns in growing rabbits. These benefits are largely attributed to the rich protein

content and favourable nutrient composition of LL leaves. However, inclusion levels above 10% may adversely affect rabbit health and productivity due to the presence of anti-nutritional factors (ANFs), primarily mimosine and tannins which can impair nutrient digestion, protein utilization, and metabolic functions. Therefore, careful management of dietary inclusion rates is required. Future research should focus on improving detoxification and processing methods, including sun-drying, soaking, fermentation, ensiling, microbial

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degradation, chemical treatment and enzyme supplementation, to reduce ANFs and maximize safe utilization of LL leaves. This review synthesizes findings from studies published over the past 15 years on the effects of LL leaves supplementation on the production performance and productivity of growing rabbits.

Keywords: Anti-nutritional factors, economic efficiency, hycote rabbit, *Leucaena leucocephala*, production performance

INTRODUCTION

Globally, the meat production sector, primarily dominated by poultry, cattle, and swine, has continued to increase and is projected to grow further in the coming years (Hayat et al., 2023). Within this sector, rabbit farming has gained growing attention due to its significant contribution to food security, income generation and sustainable agriculture. Livestock production contributes nearly 40% of global agricultural income and supplies approximately one-third of the world's total protein consumption, thereby playing a significant role in combating malnutrition and improving global food security (Naqvi et al., 2025). Rabbits are as highly efficient meat-producing animals because of their fast growth rate, early sexual maturity, high reproductive capability, prolific breeding ability and superior feed conversion (Ghosh et al., 2008). In addition, rabbit meat is vastly nutritious and widely considered a healthy source of animal protein for humans due to its favourable nutritional composition (Kumar et al., 2024). Despite these benefits, rabbit meat consumption remains low in many regions, mostly because of inadequate market availability, insufficient culinary diversification, and relatively high retail prices (Idris et al., 2023).

Nevertheless, global production of rabbit meat continues to increase, with projections estimating output to reach nearly 1.8 million tonnes by 2025. China is projected to remain the leading producer, accounting for almost 62% of total global production (Mohd Aminuddin et al., 2023). In rabbit production, growth performance is vigorously influenced by feed quality, as dietary composition directly impacts feed intake, nutrient digestibility and overall productivity. Optimal growth is usually attained with diets containing 16-18% crude protein and 14-18% crude fibre (Atmaja et al., 2024).

Rising feed expenses and consumer demand for healthier meat products have strengthened the pursuit for sustainable and cheap alternative feedstuffs for livestock production (Almahallawi et al., 2024; Cho et al., 2024; Hasan et al., 2025; Usman et al., 2025). Among the potential alternatives, LL, a tropical leguminous shrub, has gained substantial attention because of its high nutritional content and adaptability. Its leaves contain 20%-30% crude protein and 12%-20% crude fibre on a dry matter basis, making it an important feed resource for rabbits and ruminants. In addition, the LL plant is characterised by constant year-round availability, fast growth, better drought tolerance and large biomass production, further improving its suitability as a forage crop.

Previous studies have presented that dietary inclusion of LL leaves can positively improve nutrient absorption, growth, and overall feed efficiency in rabbits (Lamidi & Akilapa, 2013; Makinde, 2016). Properly processed LL leaf meal has been described to improve dry matter consumption and weight gain while lessening the adverse effects of ANFs (Al-Amin et al., 2019; Debnath et al., 2016). These findings recognised that LL leaves have potential as an alternative nutrient source in rabbit nutrition.

However, higher consumption of LL leaf meal in rabbits is limited by the anti-nutritional compounds, which adversely affect growth, nutrient digestibility, feed intake, and overall health. Fayemi et al. (2011) stated that rabbit feed containing LL leaves with ANF concentration exceeding 1% led to low feed consumption and reduced growth. Similarly, consumption of unprocessed or untreated LL leaves has been linked to poor growth response and low feed conversion, basically due to the detrimental impacts of ANFs (Fayemi et al., 2011).

Anti-nutritional compounds restrict the broader application of LL leaves in both monogastric herbivore and ruminant feeding systems (Ahmed & Abdelati, 2009; Nursiwi et al., 2018). These chemicals depress protein and dry matter digestion, thereby diminishing nutrient absorption and the efficiency of feed utilisation. Furthermore, the proportion of dietary inclusion plays a significant role in determining animal productivity and performance. Excessive inclusion levels may compromise animal health status and productivity, suggesting the necessity for carefully optimised feeding plans (Ahmed & Abdelati, 2009; Fayemi et al., 2011).

Nevertheless, variations in reported findings concerning the optimal inclusion levels, digestibility and toxicity of LL leaves continue to restrict their application in rabbit production. Therefore, this review critically assesses the potential of LL leaves as an underutilised feed supplement for rabbits by evaluating their nutritional structure, recommended inclusion rates, limitations, benefits and implications for growth response, economic sustainability, nutrient utilisation efficiency and animal health.

Rabbit Digestive Tract and Function

The mechanism of fibre digestion in rabbits is illustrated in Figure 1. Rabbits are monogastric animals with a digestive system modified for the efficient utilisation of fibrous plant parts. A typical significant structure of the rabbit digestive tract is the enlarged hindgut, called the caecum, which functions as the main site of microbial fermentation (Varga, 2014). The caecum holds a dense population of microorganisms that ferment nutrients and fibrous fractions that escape digestion and absorption in the small intestine (Gidenne et al., 2002).

Digestion process in rabbits begins in the mouth, as in other mammals. Unlike ruminants, rabbits have a deficiency of enzymes needed to completely digest structural carbohydrates such as cellulose. As digesta pass via the gastrointestinal tract, fibrous

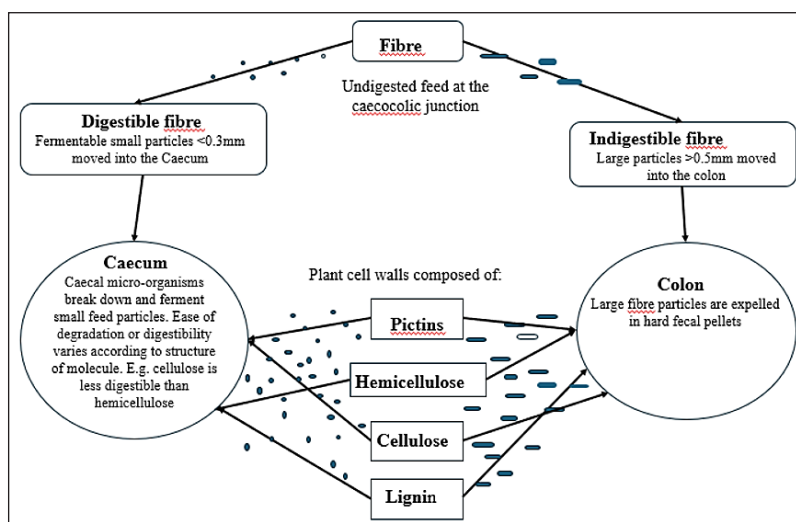


Figure 1. Mechanisms of fibre digestion in rabbits (Varga, 2014)

materials are selectively separated in the colon into digestible and indigestible portions. The digestible fibre, which holds fermentable nutrients, is redirected to the caecum, where bacterial fermentation breaks it down and releases valuable nutrients. In contrast, the indigestible fibre is compressed into hard faecal pellets and expelled as waste (Blas et al., 2020).

Inside the caecum, microbial fermentation releases crucial nutrients, including microbial proteins, volatile fatty acids (VFAs) and vitamins. These fermentation products are wrapped into soft, mucus-coated pellets commonly known as cecotropes. These cecotropes are ejected and immediately re-ingested by the rabbit via a process called coprophagy or cecotrophy. Re-ingestion permits nutrients synthesised in the caecum to move via the stomach and be efficiently absorbed in the small intestine. Volatile fatty acids produced during caecal fermentation contribute about 40% of the rabbit's daily energy supplies and play a key role in preserving caecal health. The production of VFAs helps keep the caecal pH slightly acidic, approximately 6.0-7.0, creating favourable environments for useful microorganisms while inhibiting the growth of detrimental bacteria (Gidenne & Fortun-Lamothe, 2002). This highly effective digestive approach enables rabbits to thrive well on high-fibre forage diets and effectively utilise plant parts that many other livestock species cannot.

Nutritional Composition and Requirements for Growing Rabbits

The nutritional needs of growing rabbits for optimal performance and health, presented in Tables 1 and 2, suggest that LL supplementation has the potential to meet these nutritional requirements. Their efficient inclusion in rabbit feed requires proper balancing,

Table 1
Nutritional requirements for optimum productivity in rabbits (Lebas, 2004)

Nutrients		GROWTH		REPRODUCTION		Single Feed
		18 to 42 days	42 to 75 - 80 days	Intensive	Semi-intensive	
Digestible Energy	(Kcal/kg)	2300	2500	2600	2500	2300
	(MJ/kg)	9.50	10.50	11.00	10.50	9.50
Crude Protein		151-161	161-171	182-191	172-176	161
Digestible Protein		111-121	122-132	131-141	121-131	111-126
Ratio Digest. Protein	(g/1000kcal)	46	49	52-54	51-53	49
Ratio Digestible Energy	(g/1 MJoule)	10.70	11.50	12.70-13.00	12.00-12.70	11.50-12.00
Lipids		21-26	26-41	41-51	31-41	21-31
Amino Acids						
Lysine		7.50	8.00	8.50	8.20	8.00
Sulfur amino acids (methio+cyst)		5.50	6.00	6.20	6.00	6.00
Threonine		5.60	5.80	7.00	7.00	6.00
Tryptophan		1.20	1.40	1.50	1.50	1.40
Arginine		8.00	9.00	8.00	8.00	8.00
Minerals						
Calcium		7.00	8.00	12.00	12.00	11.00
Phosphorus		4.00	4.50	6.00	6.00	5.00
Sodium		2.20	2.20	2.60	2.50	2.20
Potassium		<15	<21	<19	<19	<19
Chloride		2.80	2.80	3.50	3.50	3.00
Magnesium		3.00	3.00	4.00	3.00	3.00
Sulphur		2.50	2.50	2.50	2.50	2.50
Iron (ppm)		51	51	101	101	81
Copper (ppm)		6	6	11	11	10
Zinc (ppm)		26	26	51	51	41
Manganese (ppm)		8	8	13	13	11
Vitamins (Fat-soluble)						
Vit. A (UI/kg)		6001	6001	10001	10001	10001
Vit. D (UI/kg)		1001	1001	1001(<1500)	1001(<1500)	1001(<1500)
Vit. E (mg/kg)		≥30	≥30	≥50	≥50	≥50
Vit. K (mg/kg)		1	1	2	2	2

Note. DM, Dry Matter; Kcal/kg, kilocalories per kilogram; MJ/kg, megajoules per kilogram; g/1000 kcal,grams per 1000 kilocalories; g/1 MJoule, grams per megajoule

Table 2

Nutritional requirements for optimum health condition in rabbits (Lebas, 2004)

Nutrients	GROWTH		REPRODUCTION		Single Diet
	19 to 43 days	43 to 76-81 days	Intensive	Semi-intensive	
ADF	≥191	≥171	≥136	≥151	≥161
ADL	≥56	≥51	≥31	≥31	≥51
ADF Minus ADL (Cellulose)	≥131	≥111	≥91	≥91	≥111
Ratio ADL/Cellulose	≥0.41	≥0.41	≥0.350	≥0.400	≥0.400
NDF	≥32	≥310	≥300	≥315	≥310
NDF - ADF (Hemicellulose)	≥120	≥100	≥85	≥90	≥100
Ratio (Hemicellulose + Pectins)/ADF	≤1.30	≤1.3	≤1.3	≤1.3	≤1.3
Starch	≤141	≤201	≤201	≤201	≤161
Vitamins (Water Soluble, ppm)					
Vit. C	251	251	201	201	201
Vit. B ₁	3	3	3	3	3
Vit. B ₂	7	7	7	7	7
Nicotinamid	51	51	41	41	41
Pantothenic Acid	21	21	21	21	21
Vit. B ₆	3	3	3	3	3
Folic Acid	6	6	6	6	5
Vit. B ₁₂	.0.010	0.010	0.010	0.010	0.010
Choline	201	201	101	101	101

Note. Abbreviations: DM (Dry Matter), ADF (Acid Detergent Fibre), ADL (Acid Detergent Lignin), NDF (Neutral Detergent Fibre), ppm (parts per million)

detoxification and processing to lessen the adverse effects of ANFs. Growing rabbits need a well-balanced nourishment to support fast growth, effective nutrient utilisation, and stable physiological well-being. Digestible energy, ranging from 2300 to 2600 kcal/kg, is critical for maintaining metabolic activities and improving weight gain. Crude protein content of about 15-19% are required for muscle development, enzyme synthesis and tissue repair.

Dietary fibre, preferably between 13 and 18%, plays a crucial role in sustaining digestive health by influencing hindgut fermentation, stimulating gut motility, and lowering the risk of gastrointestinal disorders. Dietary fats serve as energy sources, boost feed palatability, and help the absorption of fat-soluble vitamins. Vitamins, namely A, D, E, and the B complex group, are essential for metabolic regulation, immune function and appropriate bone and tissue development. Minerals, including phosphorus and calcium, are indispensable for physiological balance, skeletal development and normal body functions. Sufficient water intake is equally significant, as it supports digestion, thermoregulation, nutrient transport and overall metabolic activities. Collectively, these nutrients essentially contribute to optimal growth response, increase feed efficiency, enhance health condition and better disease resistance.

Comparative Assessment of LL-Supplemented Diet and a Traditional Alfalfa-Based Diet in Rabbit Feed

Leucaena leucocephala leaves have attracted considerable interest as an alternative protein-rich forage for rabbit nutrition due to their high nutritional profile and adaptability to tropical environments. Several studies have compared the nutritional characteristics and feeding value of LL with the conventional alfalfa-based diet commonly used in rabbit production. Evidence indicates that LL can partially or completely replace alfalfa in rabbit diets without adversely affecting growth performance, nutrient utilisation, or health status when included at appropriate levels. A comparative summary of the nutritional and practical characteristics of LL-supplemented and traditional alfalfa-based rabbit diets is presented in Table 3.

Leucaena leucocephala

Leucaena leucocephala plant (Figure 2), commonly referred to as the “miracle tree,” belongs to the Mimosoideae subfamily within the Fabaceae family, which encompasses roughly 50 species of shrubs and trees (Zayed et al., 2018). Owing to its rich nutritional composition and long lifespan, LL has obtained widespread acknowledgement. *Leucaena leucocephala* forage is globally distributed (Table 4) and widely cultivated across tropical and subtropical areas, with a projected global cultivation area of 2 to 5 million hectares of land (Zayed et al., 2018).

Native to Mexico and Guatemala, LL was introduced to the Philippines and subsequently spread across Southeast Asia in the early 16th century. By the late 19th century, its cultivation had extended further across the Asia-Pacific area and into Australia. Relatively, the LL species is easy to establish and is recognised for its high DM productivity, ranging from 3 to 30 tonnes per hectare yearly. However, variation in yield is strongly affected by environmental variables such as temperature, moisture availability and soil fertility level (Al-Amin et al., 2019). For example, the cultivar OFI 34/92 has been stated to produce about 6 tonnes of DM per hectare each year when planted at a spacing of 1.5 m × 0.25 m.

Management practices also affect productivity and overall performance. Cutting LL plants at a height of 100 cm tall can yield up to 24 tonnes of DM per hectare per annum, while a 10-week harvesting interval may surge production to around 25.7 tonnes per hectare. In addition, soil amendment with gypsum has been revealed to boost edible biomass output, increasing yield to 4.5 tonnes per hectare per annum in comparison to 2.3 tonnes without gypsum (Nakamanee et al., 2019).

Leucaena leucocephala survives well in tropical and subtropical climatic areas between 30°N and 30°S (Xuan et al., 2006), performing significantly at temperatures between 25 °C and 30 °C. Its growth response declines in cooler subtropical winter regions due to restricted cold tolerance. It does not thrive well under heavy shade, but is more shade-

Table 3
Comparative assessment of an LL-supplemented diet and a traditional alfalfa-based diet in rabbit feed (Adekojo S et al., 2014; Debnath et al., 2016)

Variables	Diets Supplemented with Leucaena Leaves	Traditional Alfalfa-Supplemented Diets	Main Functions / Implications
Crude Protein (CP)	Contains high protein levels (20-30%), similar to alfalfa diets	Rich in protein (17-22%)	Supplies protein required for growth and tissue development
Fibre Content (NDF/ADF)	Higher lignin concentration with slightly lower fibre digestibility	Better digestibility of fibre and promotes intestinal health	Provides fermentable fibre that supports caecal fermentation
Nutrient Digestibility (CP, DM)	Generally, slightly lower digestibility of nutrients	Higher digestibility of protein, dry matter, and energy	Enhances nutrient availability and energy supply
Growth Performance	Like alfalfa, when included at moderate rates ($\leq 10\%$), but declines at higher levels	Consistently supports superior growth performance	Improves body weight gain, FCR and feed consumption
Feed Conversion Efficiency (FCR)	Better efficiency at low inclusion (around 10%), but poorer at higher levels	More stable and efficient feed conversion	Indicates feed utilisation efficiency and rate of growth response
Anti-Nutritional Factors (ANFs)	Contains mimosine and tannins; high levels may cause toxicity	Very low anti-nutritional compounds and safer for feeding	High ANFs reduce growth and nutrient use; low levels minimise toxicity
Palatability	Moderate acceptance, reduced at high inclusion rates	Highly palatable	Influences voluntary feed intake
Health Effects	Safe at low to moderate inclusion if properly processed; risks increase at high levels	Widely accepted and considered safe	Lower ANFs improve health and performance
Economic Efficiency	Considerably reduces feed cost	More expensive, especially in tropical areas	Can lower feeding cost to 53% at 10% inclusion rate
Recommended Inclusion Level	Around 10%; potentially higher with effective processing	Commonly used at 30-40% of the diet as a forage source	Help optimise productivity and performance
Environmental Adaptability	Well-suited to tropical and subtropical climates	Performs best in temperate environments	Affects sustainability, availability, and production cost

ADF: Acid Detergent Fibre; NDF: Neutral Detergent Fibre; DM: Dry Matter



Figure 2. *Leucaena leucocephala* plants (Nakamanee et al., 2019)

Table 4
Global Leucaena distribution (Zayed et al., 2018)

Region	Countries
Central America	Costa Rica, Mexico, Nicaragua, Guatemala, Panama, El Salvador, Honduras
Asia	Philippines, Thailand, Taiwan, India, Cambodia, Bhutan, Indonesia, Laos, Vietnam, Malaysia, Pakistan, Sri Lanka, Iran, Japan, Iraq
Australasia	Papua New Guinea (New Guinea, New Britain, Bismarck Archipelago), Australia
Caribbean	Dominican Republic, Jamaica, Haiti, Cuba, Bermuda, Puerto Rico, Grenada, Cayman Islands, Bahamas
Indian Ocean	Seychelles (Aldabra, Rodrigues, Islands), Madagascar, Mauritius, Christmas Island, Chagos Archipelago
Africa	Malawi, Kenya, Nigeria, Somalia, Ghana, Zimbabwe, Uganda, Sierra Leone, Sudan, Togo, Senegal, Angola, Liberia, Guinea, Chad, Guinea-Bissau, Niger, Mali, São Tomé and Príncipe, South Africa,
Middle East	Yemen, Saudi Arabia,
Europe	Spain
North America	United States (Arizona, Hawaii, Virgin Islands, Texas, Florida), Georgia
Pacific Ocean	Polynesia (Tahiti, Moorea), Fiji (Caroline Islands),
South America	Peru, Venezuela, Chile, Brazil, Argentina, Bolivia, Colombia, Guyana

tolerant compared to many other tree legumes (Verma, 2016). It grows well in humid to subhumid environmental conditions with yearly rainfall ranging from 650 mm to 3000 mm and can endure dry seasons lasting 4 to 6 months. It exhibits a mean tolerance to salinity, frost and temporary waterlogging (up to about three weeks). Its growth declines under adverse conditions like acidic soils ($\text{pH} < 5.5$), severe frost, and soils deficient in nutrients like phosphorus, potassium and calcium. Furthermore, it is susceptible to elevated salinity and high aluminium concentrations, both of which can markedly deteriorate its growth performance.

Effects of Dietary Inclusion of LL on Microbial Dynamics, Fermentation, and Mimosine Toxicity

Incorporation of LL leaf in rabbit feed creates a dose-dependent trade-off between nutritional enhancement and constraints by anti-nutritional compounds, with significant implications for toxin metabolism, hindgut fermentation and microbial ecology. At mean inclusion rates, LL can support cecal fermentation by providing fermentable protein and fibre portions that enhance microbial substrate availability and VFAs production. Yet, increasing supplementation rates decreases fermentation efficiency mainly because of ANFs, particularly tannins and mimosine, which can bind dietary proteins and carbohydrates, inhibit microbial enzyme activities, and lower overall nutrient availability and digestibility.

Dietary inclusion of LL can also alter the cecal microbial ecosystem significantly. Initial exposure to the LL diet may suppress sensitive bacterial populations, causing momentary drops in microbial activity and growth response. Partial microbial adaptation may occur over time, reflected in the regaining of the fermentation role. This adaptation is limited in rabbits compared to ruminants. Rabbits lack specialised and efficient microbial groups capable of degrading the toxic metabolites of mimosine, including 2,3-dihydroxypyridine (2,3-DHP) and 3,4-DHP, leading to limited detoxification capability and accumulation of toxic substances.

When inclusion rate exceeds safe thresholds (commonly reported beyond 20% dietary inclusion or higher mimosine/tannin consumption), adverse consequences such as hepatic dysfunction, alopecia and increased mortality have been documented (Fayemi et al., 2011). Prominently, subclinical conditions may still arise at lower levels of inclusion, with histopathological changes in key organs detected even without obvious clinical signs (Carlos Machado et al., 2012). Processing approaches like heat treatment and ensiling can partly mitigate toxicity by lowering mimosine and tannin concentration, thereby enhancing digestibility and fermentation performance. Overall, the addition of LL in rabbit feed is restricted by its anti-nutritional profile and the low microbial detoxification capability of the hindgut ecosystem. Efficiency of nutrient utilisation therefore, relies on careful dose optimisation and proper feed processing methods to balance improved supply of nutrients against risks of microbial inhibition and systemic toxicity.

Nutritional Content of *Leucaena leucocephala* Foliage

The nutritional content of different LL species mentioned in Table 5 demonstrated that LL foliage is a fibrous, highly nutritious forage widely recognised as a valuable feed ingredient for ruminants and monogastric herbivores (Montoya-Flores et al., 2020). Across various species and cultivars, its crude protein constituent typically ranges from 18% to 28%, demonstrating its potential as a plant-based source of protein (Nakamanee et al., 2019). In addition to its rich protein concentration, LL leaf meal comprises essential amino acids,

Table 5
Nutritional content of different LL species (Nakamanee et al., 2019)

Species	% (Dry Matter)				
	CP	ADF	NDF	Lignin	Hemicellulose
<i>Leucaena</i> (cv. Tarramba K636)	33.50	19.70	33.50	10.90	13.80
<i>Leucaena</i> (ssp. <i>glabrata</i> cv.) Cunningham	34.70	23.70	34.70	8.20	11.10
<i>Leucaena</i> (ssp. <i>zacapana</i> OFI 56/88)	35.00	23.20	35.00	6.40	11.90
<i>Leucaena</i> (OFI 52/88)	36.20	25.50	36.20	8.50	10.70
<i>Leucaena</i> (OFI 83/92)	34.10	23.00	34.10	10.30	11.10
<i>Leucaena</i> (ssp. <i>stenocarpa</i> 1 OFI53/88)	38.00	27.00	38.00	11.40	11.00
<i>Leucaena</i> (ssp. <i>esculenta</i> OFI 47/87)	34.70	23.30	34.70	8.60	11.40
<i>Leucaena</i> (ssp. <i>paniculata</i> 2 OFI 52/87)	38.00	24.90	38.00	10.00	13.10
<i>Leucaena lanceolata</i> (OFI 43/85)	37.90	25.00	37.90	7.70	12.90
<i>Leucaena lempirana</i> (OFI 6/91)	38.90	25.90	38.90	10.50	12.00
<i>Leucaena</i> (ssp. <i>glabrata</i> OFI 34/92)	35.90	23.90	35.90	8.60	12.10
<i>Leucaena</i> (ssp. <i>nelsonii</i> 3 OFI 47/85)	43.30	31.00	43.30	11.80	12.30
<i>Leucaena</i> (OFI 81/87)	44.00	35.40	44.00	3.50	3.60
<i>Leucaena</i> (OFI 83/87)	35.90	25.40	35.90	12.00	10.40
<i>Leucaena</i> (OFI 17/86)	42.20	29.30	42.20	10.00	12.80
<i>Leucaena</i> (ssp. <i>magnifica</i> 4 OFI 19/84)	41.50	29.80	41.50	11.00	11.70
<i>Leucaena</i> (OFI 61/88)	39.30	25.70	39.30	9.50	13.70
<i>Leucaena</i> (OFI 137/94 (CQ 3439)	36.40	26.00	36.40	10.40	10.40

vitamins and minerals, with an amino acid profile similar to soybean meal, making it a potential alternative source of feed ingredient for rabbits and other livestock, particularly in developing and tropical regions (Debnath et al., 2016).

The nutritional composition of LL is determined by plant maturity and plant part, with the CP value declining as harvest intervals increase. For example, protein content may reduce from 21% at 6 weeks of regrowth to nearby 16% at 12 weeks of cutting interval (Nakamanee et al., 2019; Zayed et al., 2018). Furthermore, this reduction emphasises the significance of optimal harvesting time to maximise nutritional output. Beyond its protein contribution, LL facilitate growth, enhances physiological performance, and improves metabolic functions due to its balanced nutrients (Wiratmini et al., 2021). Finally, its rich amino acid structure and wide availability in tropical countries place LL as a cheap, accessible protein feed supplement capable of partially or fully substituting conventional feed ingredients in the rabbit diet (Adedeji et al., 2013; Vijayakumar & Srinivasan, 2018).

Inclusion of Varying Rates of LL in Rabbit Feed

The implications of varying levels of LL leaves inclusion in the rabbit ration (Table 6) vary primarily due to the concentration of ANFs. Previous studies suggested that incorporating

Table 6
The impact of varying levels of LL leaf meal inclusion in rabbit feed

LL Inclusion (%)	Impacts on Growth Response and Health Status	Observed Outcomes	Reference(s)
0% (Control)	Represents the baseline diet with no LL inclusion. Animals maintained standard diets with normal growth, feed intake, and physiological health parameters.	Normal growth performance and health status under conventional feeding conditions.	(Adedokun Olubukola et al., 2021)
10%	Considered a safe inclusion level for optimal production performance and health status.	Improved growth response and reduced expenses on feed production.	(Debnath et al., 2016) (Makinde et al., 2015) (Lamidi & Akilapa, 2013) (Adedeji et al., 2013) (Makinde, 2016) (Al-Amin et al., 2019)
15%	Moderate inclusion level is associated with acceptable feed consumption and FCR with minimal adverse health effects.	Average nutrient utilisation, moderate growth response, and acceptable carcass yield.	(Castillo-Luna et al., 2022)
20%	Considered a potentially unsafe inclusion level if detoxification or processing is not done due to anti-nutritional factors.	Low growth response, decreased nutrient absorption efficiency, and potential health impairment.	(Fayemi et al., 2011)
25-30%	High inclusion levels are associated with significant toxicity issues, particularly in female and young rabbits, mainly due to ANFs.	Increased mortality, poor growth response, and deteriorated health status.	(Abd El-Galil et al., 2007)

LL leaves up to 10% supports optimal growth, improved feed utilisation efficiency, enhanced health condition, and economic output (Al-Amin et al., 2019; Debnath et al., 2016). At a 15% inclusion rate, rabbits exhibit moderate feed consumption and feed conversion ratio, with minor adverse health effects, signifying that this level may still support productivity without considerably compromising physiological function and well-being (Castillo-Luna et al., 2022).

Increasing LL inclusion to 20% demonstrated more noticeable risks due to higher ANF content, resulting in low feed intake, decreased weight gain, and clinical signs of toxicity and loss of hair (Fayemi et al., 2011). When the inclusion rate surpasses 20%, these negative impacts become severe, predominantly in females and young rabbits, with a sharp increase in mortality. This demonstrates a critical toxicity level where the detrimental effects of ANFs offset the nutritional benefits of LL leaves (Abd El-Galil et al., 2007). Overall, current

research findings support limiting LL inclusion level to not more than 10% in rabbit feed to optimise growth, maintain good health status and economic efficiency while minimising the hazards linked to ANFs.

Impact of LL Inclusion on Body Weight

Leaf of LL has gained increasing consideration as a sustainable dietary option for rabbits due to its superior nutritional profile and ability to influence nutrient utilisation and growth performance. Many research investigations have demonstrated that its inclusion in rabbit rations can improve weight gain while simultaneously reducing expenditure on feed. For instance, Pérez-Bautista et al. (2023) stated an average daily weight gain of 23.18 g/rabbit/day in rabbits offered LL foliage compared to those fed conventional commercial feed. Likewise, Castillo-Luna et al. (2022) found that partial substitution of wheat with LL leaves did not adversely affect growth performance or carcass yield, suggesting its suitability as a feed for rabbits. Al-Amin et al. (2019) also reported improved weight gain at 10% level of supplementation of leaf meal, while Fayemi et al. (2011) noted a significant growth response when ensiled LL leaves were incorporated into the diets.

Furthermore, Wiratmini et al. (2021) discovered that 30% inclusion rate of water-soaked LL leaf meal enhanced feed efficiency and weight gain, highlighting that appropriate processing can improve its nutritional status. However, its inclusion is constrained by the existence of ANFs, predominantly mimosine, which can depress growth if not sufficiently reduced. Certainly, studies have documented reduced final body weight when air-dried leaves were incorporated, highlighting the undesirable impact of an insufficient processing method (Adekojo et al., 2014; Hafsa et al., 2016). These conflicting findings underscore the relevance of effective detoxification techniques such as ensiling, soaking and fermentation to curtail ANF levels and optimise the quality of feed. Further research is required to establish optimal inclusion levels across various growth stages and to compare LL leaves with other protein-rich forages to better describe their role in rabbit nutrition.

Feed Intake

Feed intake in rabbits offered LL foliage is influenced by palatability, nutrient density, and ANF concentration. When appropriately managed, LL supplementation can enhance growth and feed conversion efficiency. However, its dietary inclusion must be carefully managed to obtain maximum benefits from LL-based diets. A study by Pérez-Bautista et al. (2024) involved 20 New Zealand rabbits that were assessed during the fattening stage to compare a commercial diet to diets supplemented with LL leaves. Results showed that rabbits offered LL-inclusive diets exhibited higher feed consumption (47.7-48.0 g/day) than those offered a standard commercial ration, indicating better palatability and voluntary feed consumption. Equally, Pérez-Bautista et al. (2023) found that inclusion of up to 33%

LL leaves enhanced both growth and feed intake, stressing its potential nutritional benefits when used at moderate rates.

On the contrary, findings are not fully consistent across different studies. Debnath et al. (2016), in a 56-day trial involving New Zealand White rabbits, compared a commercial basal diet to diets containing 10% and 15% LL leaf meal. The results showed that rabbits fed control feed recorded the highest daily feed intake (109 g/day), whereas those offered 10% (75.55 g/day) and 15% (83.57 g/day) inclusion rates consumed less feed. The reduction in intake suggests that higher inclusion rates of LL leaf meal negatively affected palatability and intake, likely due to the effects of ANF's content. Effective processing can mitigate these effects and promote growth and feed utilisation efficiency without compromising dry matter consumption (Wiratmini et al., 2021). The evidence from current literature indicates a delicate balance between the nutritional benefits of LL foliage and the risks connected to its ANFs. While lower inclusion can improve performance, responses differ depending on concentration level, processing technique and diet formulation.

Current investigations are basically limited to short and medium-term results, leaving gaps in understanding long-term physiological adaptation, chronic exposure to mimosine, and its broader metabolic impacts. Future work should focus on establishing safe inclusion thresholds across different growth stages and explaining mechanisms of mimosine metabolism, gut microbial variation, and nutrient utilisation efficiency.

Digestibility of Crude Protein

The efficiency of crude protein digestibility in growing rabbits fed LL leaves is primarily influenced by the plant's high protein content, alongside its substantial anti-nutritional compounds. Research findings on protein digestibility in rabbits remain inconsistent, mainly due to factors such as processing method, animal physiological status, inclusion level, and diet composition. Several studies reported a positive response of LL inclusion at lower levels. For example, Debnath et al. (2016) found improved CP digestibility at 10% inclusion level, while Carlos Machado et al. (2012) highlighted that this enhancement may be described by increased caecotrophy. Correspondingly, Parra-Almao et al. (2021) also reported higher CP digestibility of up to 85.9% in diets supplemented with LL.

In disparity, other studies have found neutral or negative effects, such as Adekojo et al. (2014), who reported lower digestibility in rabbits offered air-dried LL leaves, highlighting the influence of processing. Ikyume et al. (2019) stated no significant variations in CP digestibility when LL leaves were mixed with concentrate feed, suggesting that balanced supplementation may alleviate adverse effects. However, Aboul-Ela, et al. (2007) reported declined protein digestibility in rabbits fed LL as the main dietary protein source. Comparative research further indicates that LL usually exhibits lower nutrient digestibility than other forages like *Moringa oleifera*, although processing techniques can partially reduce ANFs and improve nutrient utilisation in rabbits (Safwat et al., 2014).

The differences in digestibility values are largely attributed to ANFs, which can harm enzyme activity, interfere with protein metabolism and lower nutrient absorption. Unlike conventional protein sources like soybean meal, *Moringa oleifera* contains lower rates of these compounds, contributing to its consistently higher protein digestibility. Despite mounting research interest, a vital limitation in the present literature is the lack of long-term research assessing chronic exposure to LL-associated ANFs and their overall effects on nutrient metabolism and the health of rabbits. Future research should therefore centre on optimising processing techniques and determining safe inclusion thresholds that lessen toxicity while maximising protein digestibility, especially at higher dietary incorporation rates.

Fiber Digestibility

The current literature on the inclusion of LL leaves in rabbit diets presents contradictory findings regarding fibre digestibility and nutrient utilisation. Leaves of LL are valued for their relatively high protein and fibre composition, making them a potential option; however, their nutritional advantages are highly dependent on inclusion rate, processing strategies, plant maturity, composition of diet and the proportion of ANFs.

Evidence indicated that rabbits, being hindgut fermenters, are physiologically adapted to fibre diets and can tolerate crude fibre rates of up to 30% in their feed (Makinde et al., 2015). Within this condition, moderate inclusion of LL leaf in rabbit feed has often been associated with normal or improved digestibility. For example, Safwat et al. (2014) found a rise in nutrient digestibility when inclusion rates rose from 30% to 40%, suggesting a threshold level where moderate substitution improves fermentative efficiency. Correspondingly, Adekojo et al. (2014) and Debnath et al. (2016) documented improved digestibility metrics at lower levels of inclusion, which may signal improved microbial adjustment and effective use of moderately incorporated LL. This positive result is not the same across different studies. Other research investigations reported neutral or reduced digestibility values with increasing LL leaf addition. Hafsa et al. (2016) and Nieves et al. (2004) found diminished nutrient and fibre (NDF and ADF) digestibility compared to the conventional forage feed, such as alfalfa-based diets, indicating depressed fibre utilisation at higher inclusion rates. This divergence is supported by Safwat et al. (2015), who discovered that while moderate addition may improve fermentation, excessive replacement of traditional forages can impair the digestion of fibre.

The discrepancy in findings may be attributed to different interacting factors. Research evidence showed that inclusion level seems to be dose-dependent, with low to medium rates supporting microbial functions while higher levels likely exceeding the digestive capability due to ANFs. Plant maturity also significantly influences fibre quality as older LL leaves contain higher lignified, indigestible fibre portions, thereby lowering

digestibility. Processing techniques critically alter nutritional yield. Diet formulation and the balance between LL and traditional forages vigorously influence the dynamics of fermentation in the hindgut. The evidence shows that fibre digestibility in LL-based rabbit feed is not inherently fixed but is highly dependent on several factors. The main limitation recognised across studies is the absence of standardised inclusion and processing guidelines, especially concerning the detoxification of ANFs. Addressing this gap involves systematic assessment of treated LL leaves under controlled, long-term *in vivo* trials to determine safe supplement thresholds that optimise nutrient utilisation and animal health. Standardisation is indispensable for converting LL leaf meal into a reliable and sustainable feed resource for rabbits.

Carcass Weight and Dressing Percentage

Inclusion of LL leaves in diets of growing rabbits has produced inconsistent results on dressing percentage, carcass yield, and organ development, mostly depending on inclusion level and form of processing the leaf meal diet. Makinde et al. (2015) found increased relative weights of metabolically active organs, principally the liver, kidney, and heart in rabbits offered diets comprising of 20% LL leaf meal. This response was considered an adaptive physiological constraint, reflecting increased detoxification process in response to anti-nutritional chemicals. Makinde (2016) further strengthened these findings, suggesting that hypertrophy of the organ may represent a compensatory mechanism to ease toxin load. Consistent with these findings, Fayemi et al. (2011) reported that clinical signs and symptoms of toxicity, including alopecia, hepatic damage, and necrotic lesions in rabbits exposed to comparable dietary inclusion rates.

Divergent studies reported minimal or no adverse physiological implications at moderate rates of inclusion. Candelaria-Martinez et al. (2021) observed insignificant alteration in visceral organ weights when LL leaf meal was added as a dietary supplement, whereas Castillo-Luna et al. (2022) demonstrated that partial substitution of wheat up to 15% LL leaves did not negatively impact carcass yield. These findings indicated that low to moderate inclusion levels may be physiologically acceptable under a controlled feeding system. However, negative effects became more visible at higher inclusion. Studies by Mtenga & Laswai (1994) and Tangendjaja et al. (1990) discovered reduced growth response and impaired carcass quality when LL incorporation upsurges from 40-60%, ascribing these effects primarily to ANFs. The literature outlined a clear dose-dependent response; nonetheless lacks consensus on a universally safe inclusion threshold. The literature highlights a critical gap in defining the optimal inclusion rate for LL leaf in rabbit nutrition. Most existing findings are short-term and do not sufficiently include cumulative toxicological impacts or long-term physiological adjustment. Additionally, there remains insufficient agreement on the most effective detoxification approaches or on dietary plans

capable of decreasing anti-nutritional impacts. Future studies should be directed at long-term feeding experiments, systematic evaluation of supplementation levels, and integrated mitigation approaches such as fermentation, chemical and biological detoxification, and genetic selection for low-mimosine varieties. These approaches are vital to improving the safety and nutritional reliability of LL as a sustainable feed resource in rabbit production.

Economic Efficiency of LL Leaves Inclusion

Leucaena leucocephala leaf incorporation into rabbit rations has been associated with improved growth rate and lower feeding costs, largely due to its relatively high CP content and potential to partly substitute for more costly commercial feed ingredients. Research by Wiratmini et al. (2021) reported higher weight gain and better feed efficiency without adverse effects on DM intake, indicating efficient nutrient absorption. Similarly, Candelaria-Martinez et al. (2021) discovered that combining LL leaves with *Guazuma ulmifolia* foliage depressed carcass fat deposition while preserving meat yield, suggesting its utilisation for the production of lean meat. Moreover, Debnath et al. (2016) found substantial economic returns, involving a 53% reduction in the cost of producing 1 kg of live weight when LL was incorporated at 10% of the diet, highlighting its strong short-term economic benefits.

Notwithstanding, the commercial application of LL leaves is hindered by the presence of ANFs, which can cause toxic effects if inadequately handled. Nevertheless, findings suggest that simple processing strategies, such as soaking, can significantly diminish mimosine content and associated toxicity dangers (De Angelis et al., 2021). Corresponding findings by Sarkar et al. (2021) indicated that processing appropriately and controlled dietary supplementation can mitigate ANF's effects while preserving nutritional value in both rabbits and other species of livestock. Yet, these benefits must be measured against additional operational expenses associated with detoxification, processing infrastructure and labour, which may limit scalability in commercial production systems.

A key limitation in the present literature is the insufficient data on long-term comprehensive economic assessment that integrates productivity output with processing and management expenditure. Inconsistency in LL nutritional composition driven by geographic, seasonal and post-harvest processing variances further complicates the constant economic assessment process (De Angelis et al., 2021). Thus, its true value as a justifiable feed ingredient remains dependent on context. Further research should therefore explore multi-location and multi-season experiments, alongside standardised assessment of detoxification systems and inclusion levels tailored to special rabbit breeds and production systems. Additionally, robust cost-benefit models integrating labour, availability of local feed and processing inputs are indispensable to determine the actual commercial feasibility of LL-based feeding strategies. Overall, though LL leaves show strong potential as a cost-effective and nutritionally viable feed supply for rabbit farming, its widespread

implementation depends on resolving critical constraints related to cost variability, toxicity management and system-specific performance results. An integrated evidence-based merging of nutritional and economic data is essential to establish standardised protocols for its safe and profitable inclusion in rabbit feed.

The Anti-nutritional Compounds in LL

The rate of ANFs and the dry matter digestibility percentage of different LL cultivars is presented in Table 7. The ANFs commonly found in LL leaves that adversely affect rabbit production performance include tannins and mimosine. Tannins are polyphenolic compounds known to interfere with nutrient absorption and metabolic processes, whereas mimosine is a non-protein amino acid which can disrupt protein synthesis and DNA replication. These compounds contribute to impaired growth, reduced feed intake and toxicity. A study by Pal et al. (2010) revealed that mimosine impedes protein biosynthesis in rabbits, resulting in toxic indicators such as stunted growth. The toxic implications of LL supplementation have been documented in multiple species, including cattle, sheep, and rabbits, while in goats, clinical signs like alopecia have been detected (Vijayakumar & Srinivasan, 2018). In rabbits specifically, extreme inclusion of LL leaves has been associated with limited growth response (Adedeji et al., 2013). Feed containing higher levels of LL leaves disrupts metabolic processes, causing alopecia and feed-related toxicity in rabbits (Al-Amin et al., 2019). Inclusion rate of up to 20% LL has been reported to induce severe alopecia in rabbits (Makinde et al., 2015), substantiating earlier findings by D'mello (1992). The post-mortem investigations of affected animals further discovered pathological lesions such as necrosis, hepatic congestion and oedema, supportive of earlier evidence of tannin-induced and mimosine organ impairment (Awosanya & Akinyode, 1995).

Tannins form stable tannin protein complexes, diminish protein digestibility and impair growth response (Schofield et al., 2001). Tannins existing in both stems and leaves decrease DM digestibility, although they may simultaneously upsurge the proportion of caecal undegradable protein (Schofield et al., 2001). The astringent taste of tannin can also reduce voluntary feed consumption, inhibit the activity of digestive enzymes and further compromise nutrient absorption (Makkar, 2005). Leaves of LL contained relatively high content of mimosine (1.64%) and tannins (1.20%) after sun drying (Fayemi et al., 2011). At elevated inclusion levels above 20% of rabbit diets, these leaves have been linked to severe health outcomes, including hepatic dysfunction, alopecia, reduced growth performance and higher mortality.

Bioactivity and Phytochemical Contents of LL Leaves

Leucaena is a leguminous plant extensively cultivated in tropical and subtropical areas and documented for its multifaceted applications in traditional medicine, agriculture and

Table 7
The rate of ANFs and DMD of different LL cultivars (Nakamanee et al., 2019)

Species	The Anti-nutritional Compounds % (DM)				%DMD (24h)
	Mimosine	%	Tannin	%	
<i>Leucaena (ssp. glabrata cv. Cunningham)</i>	Mimosine	3.2	Tannin	0.9	75
<i>Leucaena (ssp. OFI 56/88)</i>	Mimosine	2.4	Tannin	0.5	75
<i>Leucaena (OFI 52/88)</i>	Mimosine	3.2	Tannin	1.8	73
<i>Leucaena (OFI 83/92)</i>	Mimosine	2.9	Tannin	2.1	72
<i>Leucaena (ssp. stenocarpa1 OFI 53/88)</i>	Mimosine	2.1	Tannin	2.3	52
<i>Leucaena (ssp. esculenta OFI 47/87)</i>	Mimosine	1.1	Tannin	3.2	65
<i>Leucaena (ssp. paniculata2 OFI 52/87)</i>	Mimosine	1.6	Tannin	0.9	67
<i>Leucaena (OFI 43/85)</i>	Mimosine	3.1	Tannin	1.0	74
<i>Leucaena (OFI 6/91)</i>	Mimosine	2.3	Tannin	0.4	71
<i>Leucaena (ssp. glabrata OFI 34/92)</i>	Mimosine	3.3	Tannin	1.1	80
<i>Leucaena (ssp. nelsonii3 OFI 47/85)</i>	Mimosine	2.9	Tannin	0.9	53
<i>Leucaena (OFI 81/87)</i>	Mimosine	2.4	Tannin	0.5	58
<i>Leucaena pulverulenta (OFI 83/87)</i>	Mimosine	2.2	Tannin	3.4	44
<i>Leucaena (OFI 17/86)</i>	Mimosine	2.0	Tannin	0.3	69
<i>Leucaena (ssp. magnifica4 OFI 19/84)</i>	Mimosine	1.9	Tannin	0.3	68
<i>Leucaena (OFI 61/88)</i>	Mimosine	3.3	Tannin	0.3	65
<i>Leucaena (OFI 137/94 (CQ 3439)</i>	Mimosine	1.6	Tannin	2.7	59

Note. DM means Dry Matter; OFI means Oxford Forestry Institute; DMD means Dry Matter Digestibility

environmental management. Its leaves are particularly high in bioactive phytochemicals, including alkaloids, tannins, flavonoids and triterpenoids like lupeol, which collectively contribute to its antibacterial, anti-methanogenic and antioxidant activities (Elzaia et al., 2024; Shakak et al., 2025). Among LL alkaloids, mimosine is a major compound associated with antimicrobial function, where it is noted to hinder bacterial cell wall biosynthesis, principally peptidoglycan formation, ultimately causing bacterial growth inhibition and cell mortality (Rosida et al., 2017). Kaempferol-3-O-rutinoside, quercetin-3-O-rhamnoside and luteolin-7-glucoside are flavonoids broadly distributed in pods, leaves and flowers. However, these compounds apply antibacterial effects via multiple mechanisms involving disrupting membrane integrity, binding to bacterial proteins and inhibiting essential metabolic activities, while demonstrating strong antioxidant activity through the free radical scavenging process (Mohammed et al., 2015).

The tannin proportion was reported at around 6.57% in LL leaves (Mahanani et al. (2020), contribute to bactericidal properties by complexing with proteins of microbial cell wall, increasing membrane permeability disruption and depressing enzymatic activities. In ruminants, these tannins are also linked to low methanogenic archaea populations, thus contributing to decreased methane emissions and enhanced environmental sustainability

and management (Alduwish et al., 2025; Aydin, 2024; Khatoon et al., 2025). Lupeol, a triterpenoid compound, further boost the plant's bioactivity profile, demonstrating antimicrobial, anti-inflammatory and cytotoxic properties. Spectroscopic evaluation has confirmed lupeol occurrence in leaf extracts, with a UV absorption point typically observed between 210-214 nm (Zarina et al., 2017). MS-GC profiling of dichloromethane extracts has further identified compounds like lupeol (14.7%), β -sitosterol (9.1%), squalene (41%), phytol (34%), and other fatty acid methyl ester compounds (Bassey et al., 2023). Overall, these findings underscore LL leaves as a noteworthy reservoir of pharmacologically active phytochemicals with possible applications in antimicrobial healing, livestock feed optimisation, and methane mitigation tactics. Finally, further *in vivo* studies are necessary to fully elucidate and harness these bioactive compounds for agricultural and biomedical innovation.

Mimosine

Mimosine is a non-proteinogenic amino acid occurring naturally in leguminous plants, including LL and *Mimosa pudica*. It is chemically identified as β -[N-(3-hydroxy-4-oxopyridyl)]- α -aminopropionic acid and displays structural resemblance to tyrosine because of its 3-hydroxy-4-pyridone ring (Figure 3). Thus, this structural mimicry allows its interference with the synthesis of protein and key metabolic activities. The cytotoxic impacts of mimosine are primarily connected to cell cycle arrest at the G1 phase, inhibition of DNA multiplication, and interruption of metal homeostasis via strong chelation of transition metals, including iron and zinc, by this means promoting oxidative stress and triggering cell death. These mechanisms buttress its toxicological indicators, including reproductive impairment, growth retardation, cataract formation, alopecia and hepatic injury.

Mimosine, despite its toxicity, has proven notable pharmacological potential. In research involving cancer, it has been reported to impede p21-activated kinase 1 (PAK1), an oncogenic signalling kinase involved in tumour cell proliferation and survival (Nguyen & Tawata, 2016). In addition, extracts of *Mimosa pudica* comprising mimosine have

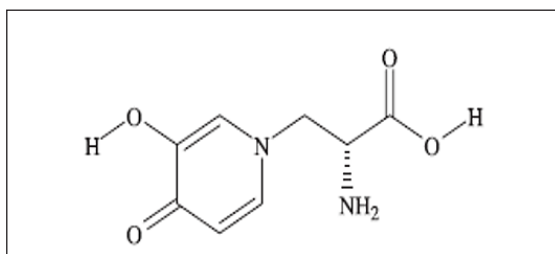


Figure 3. Chemical Structure of Mimosine (Xuan et al., 2006)

demonstrated hepatoprotective effects, likely mediated via decreased lipid peroxidation and enhanced activity of antioxidant enzymes in alcohol-induced liver injury in persons (Adurosakin et al., 2023).

The inclusion of forages comprising mimosine, such as LL, in animal feed signals imperative safety alarms, particularly for monogastric herbivores like rabbits. Gut microbial consortia in ruminants, including *Clostridium butyricum*, *Synergistes jonesii* and *Streptococcus lutetiensis*, can effectively degrade mimosine and its toxic metabolites, comprising 3,4- and 2,3-dihydroxypyridine (DHP), to less harmful compounds. This detoxification system is absent in rabbits, due to a lack of a specialised microbiota population (Derakhshani et al., 2016). Consequently, ingestion of plants high in mimosine in rabbits may result in impaired reproduction, oxidative hepatic impairment, disruption of tyrosine metabolism and reduced growth. Even though ruminant-based research continues to search for microbial adaptation and detoxification tactics for DHP and mimosine (Shelton & Brewbaker, 1994), there is a lack of recent direct investigations in rabbits. Modern-day research in the nutrition of rabbits instead centres on safe phytogetic additives like *Padina pavonica* and extruded linseed, which improve meat quality without causing adverse physiological impacts on rabbits (Fehri et al., 2025).

Current evidence does not establish the safe inclusion rate of mimosine in plants in rabbit feed. Contrary to ruminants, rabbits lack the microbial capability to detoxify mimosine and its metabolites, rendering their application physiologically and nutritionally harmful. Future research may search microbial engineering or targeted inoculation approaches to aid detoxification within the rabbit cecal environment; such approaches remain investigational. Pursuing this direction would involve integrative methodologies merging metagenomics, in vitro cecal fermentation models, and synthetic biology to identify and reconstruct appropriate detoxification pathways. Such a strategy would also necessitate a stable microbial colonisation and functional persistence inside the rabbit gut ecosystem. Mimosine-containing forages like LL will remain unsuitable for rabbit nutrition until such interventions are validated.

Tannin

Tannin is a polyphenolic phytochemical extensively present in LL leaves and seed pods. It occurs predominantly in the form of condensed tannin (proanthocyanidins), which are polymers of flavan-3-ols, even though smaller fractions of hydrolyzable tannins from gallic and ellagic acid esters may also exist. Tannin compounds exhibit a double biological profile, combining pharmacological benefits with significant anti-nutritional implications, particularly in monogastric herbivores like rabbits (Figure 4). Tannins from a functional perspective demonstrate antioxidant behaviour through scavenging reactive oxygen species and chelating transition metal ions, in so doing limiting oxidative impairment. However,

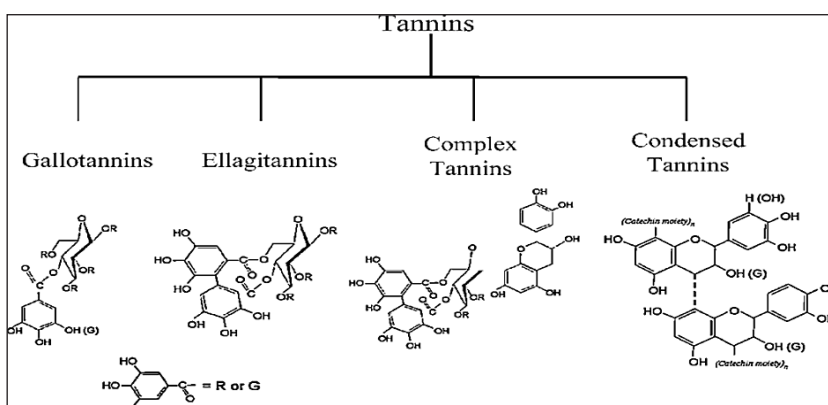


Figure 4. Tannin chemical structure (Aguilar et al., 2007)

they also display antimicrobial characteristics by disrupting microbial cell membranes and impeding significant enzymatic functions, whereas their anti-inflammatory impacts are related to suppression of lipoxxygenase action and downregulation of the pro-inflammatory cytokines process (Zarina et al., 2017). In spite of these useful properties, tannins can impair nutrient use by forming stable complexes with digestive enzymes and dietary proteins, leading to low protein digestibility and diminished growth response when dietary concentrations surpass 1% (Fayemi et al., 2011).

Excessive tannin consumption in rabbits offered LL-based diets has been associated with clinical and physiological toxicity, including retarded weight gain, poor feed conversion, hepatic congestion, alopecia, and high mortality at higher inclusion rates. Tannin content in LL is highly variable and mainly influenced by plant part, plant maturity and post-harvest processing procedures. For instance, sun-dried LL leaves might contain about 1.2% tannins, while hydroponically aged seeds showed a marked drop from roughly 9% to 1.1% after 20 days of treatment (López Valoy et al., 2012). Likewise, ensiling and hot-water treatment can further lower tannin levels to safer ranges of around 0.77-1.1%, thus improving digestibility and animal performance (Fayemi et al., 2011). Overall, tannins in LL contribute important bioactive properties and also present significant anti-nutritional dangers when present in excess. Appropriate processing strategies, namely ensiling, fermentation and thermal treatments, can substantially reduce tannin concentration, thereby improving the safety and nutritional efficacy of LL as a high-protein feed resource with agricultural and pharmacological relevance.

Methods Used to Lower Anti-Nutritional Factors Content in LL

Different processing strategies have been examined to reduce ANFs, particularly mimosine and tannins, in LL leaves intended for rabbit feeding, each varying in efficacy, nutrient conservation, and practical viability (Table 8). Thermal processing, like boiling, can partly

Table 8
Methods for minimising anti-nutritional compounds

Method	Impacts on ANFs	References
A combination of LL with other forages	Mixing <i>Leucaena</i> with other forage materials can reduce the effective concentration of anti-nutritional compounds through dilution, thereby lessening their harmful impacts on rabbits. But this approach does not fully eliminate the toxins.	(De Angelis et al., 2021) (Bernardino et al., 2021) (Pérez-Bautista et al., 2023)
Sun drying	Sun drying can cause a minor decrease in ANFs through exposure to sunlight and heat, although its effect on mimosine is limited. It may also improve palatability while maintaining much of the nutrient content.	(Adekojo et al., 2014) (Silva et al., 2024) (Agbo et al., 2017) (Debnath et al., 2016)
Soaking in normal H ₂ O	Soaking in fresh water helps lower mimosine and other water-soluble anti-nutritional compounds. This treatment may also modestly improve nutrient utilisation.	(Adekojo et al., 2014) (Wiratmini et al., 2021)
Hot H ₂ O soaking	Hot water soaking provides a greater reduction in ANFs than cold water soaking, particularly for mimosine, due to faster leaching aided by heat. Significant reductions may occur within 24 hours.	(Adekojo et al., 2014) (Fayemi et al., 2011)
Boiling	Boiling is highly effective in reducing ANFs through thermal degradation and leaching of toxic compounds. However, excessive boiling may also lead to nutrient losses.	(Agbo et al., 2017)
Ensiling	Ensiling lowers ANFs through microbial fermentation during storage. This process can substantially reduce ANFs, thereby improving growth performance, feed efficiency and rabbit health.	(Fayemi et al., 2011) (Chen et al., 2014)
Fermentation	Fermentation is considered one of the most effective detoxification methods. Beneficial microbes degrade mimosine and tannins efficiently, resulting in marked improvements in rabbit growth and overall health.	(Nursiwi et al., 2018) (Utami & Akbar, 2025)

degrade mimosine and improve digestibility, with benefits reported on improved rabbit growth (Agbo et al., 2017), though its usefulness is affected by the risk of substantial nutrient loss when exposure time is excessive, making the control process essential to avoid declining feed quality. Drying approaches are generally effective for dropping mimosine content while maintaining a better nutrient composition, alongside enhancements in the palatability of the diet. Soaking practices, involving prolonged water-soaking (36 hours) and hot-water treatment at nearly 60 °C for 24 hours, provide reasonable falls in mimosine content and can enhance intake moderately, with hot water soaking offering a more promising balance between nutrient retention and detoxification than prolonged

boiling (Adekojo et al., 2014). Fermentation efficiently reduced both tannins and mimosine, enhancing animal performance such as health condition and carcass quality, mainly in systems where regulated microbial processing is feasible.

Ensiling stands out among the more effective biological and preservation methods, for its consistent decrease of both mimosine and tannin while simultaneously improving feed efficiency and growth outcomes, particularly when done over extended periods (around 6 weeks) (Fayemi et al., 2011). The effectiveness of ensiling depends on appropriate anaerobic conditions and standardisation of the process, which may restrict adoption in low-resource locations. Blending LL leaves with alternative forage leaves can dilute ANF strengths and improve protein balance, offering low-cost but comparatively moderate detoxification impact. Long-term genetic improvement of LL cultivars can also reduce the ANF level. Overall, these methods can be classified from lower to higher effectiveness in ANFs mitigation and productivity gains from forage blending to fermentation. Selection of a suitable method should therefore be determined by operational scale, infrastructure, and the needed balance involving detoxification efficiency and nutrient preservation.

Contributions to the Rabbit Industry

Leucaena leucocephala leaves are a potential low-cost alternative feed resource for rabbit farming, specifically in tropical and resource-scarce regions where traditional protein sources are expensive or limited. The high crude protein concentration and digestibility value support the use of LL leaves as a partial replacement for commercial feed ingredients to enhance production efficiency.

Research evidence indicates that LL supplementation can increase rabbit performance and health status. Pérez-Bautista et al. (2023) found higher feed consumption and growth response in rabbits fed fresh LL leaves compared to those on commercial diets alone, suggesting positive impacts on voluntary feed intake and weight gain. Economically, LL inclusion may reduce reliance on soybean meal and alfalfa in commercial concentrates, thereby lowering feed costs and supporting cheap rabbit meat production, particularly for smallholder farmers.

However, the practical utilisation of LL remains limited by anti-nutritional compounds, which impair animal productivity and health at high inclusion levels. The variability in findings among different studies also indicated inconsistent animal responses and highlights the necessity for safe inclusion thresholds, standardised feeding strategies and effective detoxification methods.

Overall, LL leaves inclusion showed strong potential as a sustainable and economical feed resource for rabbit production based on the literature; however, their successful application depends on various factors, which include balanced dietary inclusion and further long-term study on productivity and safety.

CONCLUSION

This critical review of LL leaves incorporation into rabbit feed recommends that an inclusion level of 10% appears optimal for enhancing production performance. At this level of inclusion, significant improvements are observed in feed efficiency, body weight gain and nutrient utilisation, alongside a potential decrease in feed cost and improved health benefits. However, exceeding 10% inclusion threshold without effective and appropriate processing or detoxification may compromise nutrient utilisation and result in adverse physiological implications, including organ dysfunction in the liver, heart and kidneys. Clinical symptoms such as necrotic lesions and alopecia have also been reported under higher inclusion. These negative outcomes were primarily attributed to anti-nutritional compounds in LL foliage, specifically mimosine and condensed tannins, which interfere with metabolic activities and nutrient absorption. Therefore, careful management of the inclusion level is necessary to balance nutritional benefits with safety concerns. Further research should focus on the development and validation of efficient detoxification or processing methods, as well as strategies to boost digestibility and decrease anti-nutrient activity. Additionally, long-term feeding trials and assessments of LL in combination with other forage feedstuffs across varying inclusion gradients are required to establish robust and context-specific feeding standards. Overall, optimised use of LL foliage could offer a cost-effective feed resource, contributing to decreased production expenses, improved farmer profitability and boosting the availability of rabbit meat, particularly in tropical and low-resource agricultural regions.

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REFERENCES

- Abd El-Galil, K., Soliman, M. M., Attia, A., & Abd El-Dayem, A. (2007). Using *Leucaena* leaf meal in doe rabbit diets. *Journal of Agricultural Sciences, Mansoura University*, 32(8). <https://doi.org/10.21608/jappmu.2007.219808>
- Aboul-Ela, S., Abd El-Galil, K., Soliman, M., Attia, A., & Abd El-Dayem, A. (2007). Using *Leucaena* leaf meal in doe rabbit diets. *Journal of Animal and Poultry Production*, 32(8), 6225-6237. <https://doi.org/10.21608/jappmu.2007.219808>
- Adedokun Olubukola, O., Ibe, P. C., Safiyu, K. K., & Onabanjo, R. S. (2021). Growth performance, carcass characteristics and relative organ proportions of weaner rabbits fed diets containing graded levels of *Leucaena leucocephala* leaf meal. *Malaysian Journal of Animal Science*, 24(2), 31-38.

- Adedeji, O. S., Amao, S. R., Ameen, Adedeji, T. A., & Ayandiran, T. A. (2013). Effects of varying levels of *Leucaena leucocephala* leaf meal diet on the growth performance of weaner rabbit. *Journal of Environmental Issues and Agriculture in Developing Countries*, 5(1), 5-9. <https://icidr.org.ng/index.php/jeiadc/article/view/220>
- Adekojo, Adama, T., & Ijaiya, A. (2014). Effects of different methods of processing *Leucaena leucocephala* leaf meal on growth performance and nutrient digestibility of rabbits. *International Journal of Agriculture and Forestry*, 4(5), 380-385. <https://doi.org/10.5923/j.ijaf.20140405.06>
- Adekojo S, Adama T, Ijaiya A, & Owoleke O. (2014). Effects of dietary inclusion of differently processed *Leucaena leucocephala* leaf meal on carcass characteristics of rabbits (*Oryctolagus cuniculus*). *International Journal of Food Science and Nutrition Engineering*, 4(5), 118-127. <https://doi.org/10.5923/j.food.20140405.02>
- Adurosakin, O. E., Iweala, E. J., Otike, J. O., Dike, E. D., Uche, M. E., Owanta, J. I., Ugbogu, O. C., Chinedu, S. N., & Ugbogu, E. A. (2023). Ethnomedicinal uses, phytochemistry, pharmacological activities and toxicological effects of *Mimosa pudica*—A review. *Pharmacological Research - Modern Chinese Medicine*, 7, Article 100241. <https://doi.org/10.1016/j.prmcm.2023.100241>
- Agbo, A., Balogun, J., Oniye, S., & Auta, J. (2017). Effect of different processing methods on nutritional composition of *Leucaena leucocephala* (Lam De Wit) leaves as inclusion in fish feed. *Journal of Applied Sciences and Environmental Management*, 21(4), 719-725. <https://doi.org/10.4314/jasem.v21i4.12>
- Aguilar, C. N., Rodríguez, R., Gutiérrez-Sánchez, G., Augur, C., Favela-Torres, E., Prado-Barragan, L. A., Ramírez-Coronel, A., & Contreras-Esquivel, J. C. (2007). Microbial tannases: Advances and perspectives. *Applied Microbiology and Biotechnology*, 76, 47-59. <https://doi.org/10.1007/s00253-007-1000-2>
- Ahmed, M. E., & Abdelati, K. A. (2009). Chemical composition and amino acids profile of *Leucaena leucocephala* seeds. *International Journal of Poultry Science*, 8(10), 966-970. <https://doi.org/10.3923/ijps.2009.966.970>
- Al-Amin, N., Astuti, A., & Widi, T. S. M. (2019). Nutrient intake and performance of male New Zealand White rabbits fed different level of *Leucaena* leaf meal in pelleted complete diets. *IOP Conference Series: Earth and Environmental Science*, 387, Article 012105. <https://doi.org/10.1088/1755-1315/387/1/012105>
- Alduwish, Alharthi, Alharbi, Albalawi, Obidan, Beyari, Al-Gheffari., Shakak, Allohlubi, Almutairi, Almasoudi, Alsulami, Al-Doaiss, & Abu-Elsaoud. (2025). Evaluating the multifunctional therapeutic potential of phycocyanin: Antidiabetic, antioxidant, anticancer and antimicrobial effects on defense genes, metabolic, oxidative, and histopathological parameters in dithizone-induced diabetic rats. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 31(5), 619-634. <https://doi.org/10.9775/kvfd.2025.34347>
- Almahallawi, R., Al-Hoshani, N., Al-Nabati, E. A., Althubiani, S. A., Negm, S., El-Lkott, A. F., Bajaber, M. A., Albohiri, H. H., Khan, S. A., Mahjoub, H. A., Nazish, N., Alsolami, A. M., & Ahmed, A. E. (2024). Exploring the anticoccidial, growth-promoting, hematological and serological potential activities of *Linum usitatissimum* essential oil in broiler birds. *Pakistan Veterinary Journal*, 44(1), 117-122. <https://doi.org/10.29261/pakvetj/2024.137>
- Atmaja, R. R. D., Inayatilah, F. R., Muktisari, M. Y., Ni'mah, S., & Subandi, I. (2024). The potential of red fruit oil emulgel (*Pandanus conoideus* Lamk.) in accelerate the proliferation phase of rat incision wound healing. *Journal of Medicinal and Pharmaceutical Chemistry Research*, 6(3), 302-313. <https://doi.org/10.48309/jmpcr.2024.424847.1033>

- Awosanya, B. & Akinyode, M. A. (1995). Treatment effect of *Leucaena leucocephala* leaf meal on the carcass characteristics of rabbits. *Nigerian Journal of Animal Production*, 22(1), 90-94.
- Aydin. (2024). From deadly to life-saving effects: Antimicrobial and antibiofilm effects of *Tarantula cubensis* extract on bacterial and fungal pathogens. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 31(1), 59-67. <https://doi.org/10.9775/kvfd.2024.32854>
- Bassey, R. A., Ndarake, E. I.-I., Ngele, B. A., & Iniobong, E. A. (2023). GC-MS analysis and phytochemical constituents in *Anthocleista vogelii* and *Leucaena leucocephala* obtained from Calabar, Cross River State, Southern Nigeria. *Researchers Journal of Science and Technology*, 3(2), 68-81. <https://doi.org/10.83080/rejost.vol3no2.65>
- Bernardino, C. -M., Chiquini-Medina, R. A., Angulo-Balán, O. G., Ramírez-Bautista, M. A., Cuervo-Osorio, V. D., Quetz-Aguirre, E. M., & Flota-Bañuelos, C. (2021). Productive parameters and carcass yield of rabbits supplemented with *Leucaena leucocephala* (Lam.) de Wit., and *Guazuma ulmifolia* Lam. foliage. *Agroproductividad*, 14(1). <https://doi.org/10.32854/agrop.v14i1.1708>
- Blas, C. De, García, J., Carabaño, R., Blas, C. De, García, J., Carabaño, R., & Annales, A. (2020). Role of fibre in rabbit diets: A review. *Annales de Zootechni*, 48(1), 3-13. <https://doi.org/10.1051/animres:19990101>
- Candelaria-Martinez, B., Chiquini-Medina, R. A., Angulo-Balán, O. G., Ramírez-Bautista, M. A., Cuervo-Osorio, V. D., Quetz-Aguirre, E. M., & Flota Bañuelos, C. (2021). Productive parameters and carcass yield of rabbits supplemented with *Leucaena leucocephala* (Lam.) de Wit., and *Guazuma ulmifolia* Lam. foliage. *Agro Productividad*, 14(1), 3-8. <https://doi.org/10.32854/agrop.v14i1.1708>
- Carlos Machado, L., Motta Ferreira, W., Scapinello, C., & Brasileira de Zootecnia, R. (2012). Apparent digestibility of simplified and semi-simplified diets, with and without addition of enzymes, and nutritional value of fibrous sources for rabbits. *Revista Brasileira de Zootecnia*, 41(7), 1662-1670. <https://doi.org/10.1590/S1516-35982012000700015>
- Castillo-Luna, G., Ramos Cortez, L., Camacho Morales, R. L., Avelar Lozano, E., Sáenz Flores, E., & Buenabad Carrasco, L. (2022). Follaje de leucaena (*Leucaena esculenta*) como reemplazo parcial de trigo en dietas para conejos de engorda: comportamiento productivo y rendimiento en canal [Leucaena (*Leucaena esculenta*) foliage as a partial replacement for wheat in diets of fattening rabbits: Performance and dressing percentage]. *Archivos Latinoamericanos de Producción Animal*, 30(Supl. 1), 143-145. <https://doi.org/10.53588/alpa.300517>
- Chen, X. Z., Feng, F., Liu, Q. H., & Zhang, J. G. (2014). Degrading mimosine and tannins of *Leucaena leucocephala* by ensiling. *Applied Mechanics and Materials*, 618, 349-353. <https://doi.org/10.4028/www.scientific.net/AMM.618.349>
- Cho, H. Y., Lee, G. Y., Ali, M. S., & Park, S. C. (2024). Effects of dietary intake of heat-inactivated *Limosilactobacillus reuteri* PSC102 on the growth performance, immune response, and gut microbiota in weaned piglets. *Pakistan Veterinary Journal*, 44(3), 819-825. <https://doi.org/10.29261/pakvetj/2024.224>
- De Angelis, A., Gasco, L., Parisi, G., & Danieli, P. P. (2021). A multipurpose leguminous plant for the Mediterranean countries: *Leucaena leucocephala* as an alternative protein source: A review. *Animals*, 11(8), Article 2230. <https://doi.org/10.3390/ani11082230>

- Debnath, M., Khandakar, Z. H., Alam, M. A., & Heyamet, M. A. (2016). Effect of feeding different level of *Leucaena leucocephala* (ipil ipil) leaf meal for rabbit diet. *Asian-Australasian Journal of Bioscience and Biotechnology*, 1(2), 261-267. <https://doi.org/10.3329/aaajbb.v1i2.61573>
- Derakhshani, H., Corley, S. W., & Al Jassim, R. (2016). Isolation and characterisation of mimosine, 3,4-DHP and 2,3-DHP degrading bacteria from a commercial rumen inoculum. *Journal of Basic Microbiology*, 56, 580-585. <https://doi.org/10.1002/jobm.201500590>
- D'Mello, J. P. F. (1992). Chemical constraints to the use of tropical legumes in animal nutrition. *Animal Feed Science and Technology*, 38(2-3), 237-261. [https://doi.org/10.1016/0377-8401\(92\)90105-F](https://doi.org/10.1016/0377-8401(92)90105-F)
- Elzaia, M. A., Mandour, A. S., Youssef, M. A. H., Wafa, H. A., Aljahdali, S. M., Shakak, A. O., Husnain, L. Al, Alqahtani, M. A., Alghamdi, M. A., Abuzaid, A. O., Alqahtani, T. M., Al-Gheffari, H. K., Bouqellah, N. A., & Heakel, R. M. Y. (2024). Biochemical and molecular characterisation of five basil cultivars extract for enhancing the antioxidant, antiviral, anticancer, antibacterial and antifungal activities. *Pakistan Veterinary Journal*, 44(4), 1105-1119. <https://doi.org/10.29261/pakvetj/2024.279>
- Fayemi, P. O., Onwuka, C. F. I., Isah, O. A., Jegede, A. V., Arigbede, O. M., & Muchenje, V. (2011). Effects of mimosine and tannin toxicity on rabbits fed processed *Leucaena leucocephala* (Lam) De Wit. leaves. *African Journal of Agricultural Research*, 6(17), 4081-4085. <https://doi.org/10.5897/AJAR11.32>
- Fehri, N. E., Contò, M., Castrica, M., Quattrone, A., Renzi, G., Di Giovanni, S., Agradi, S., Vigo, D., Brecchia, G., Menchetti, L., Balzaret, C. M., Beqiraj, D., Andoni, E., Curone, G., & Failla, S. (2025). Effects of diets containing extruded linseed and *Padina pavonica* algae on meat rabbit: Carcass performance and meat quality. *Foods*, 14(2), Article 274. <https://doi.org/10.3390/foods14020274>
- Ghosh, S. K., Das, A., Bujarbaruah, K. M., Asit, D., Dhiman, K. R., & Singh, N. P. (2008). Effect of breed and season on rabbit production under sub-tropical climate. *World Rabbit Science*, 16, 29-33. <https://doi.org/10.4995/wrs.2008.638>
- Gidenne, T., & Fortun-Lamothe, L. (2002). Feeding strategy for young rabbits around weaning: A review of digestive capacity and nutritional needs. *Animal Science*, 75(2), 169-184. <https://doi.org/10.1017/S1357729800052942>
- Gidenne, T., Jehl, N., Segura, M., & Michalet-Doreau, B. (2002). Microbial activity in the caecum of the rabbit around weaning: Impact of a dietary fibre deficiency and of intake level. *Animal Feed Science and Technology*, 99(1-4), 107-118. [https://doi.org/10.1016/S0377-8401\(02\)00138-4](https://doi.org/10.1016/S0377-8401(02)00138-4)
- Hafsa, A. S. H., Salem, A. Z. M., Hassan, A. A., Kholif, A. E., Elghandour, M. M. Y., Barbabosa, A., & Lopez, S. (2016). Digestion, growth performance and caecal fermentation in growing rabbits fed diets containing foliage of browse trees. *World Rabbit Science*, 24(4), 283-293. <https://doi.org/10.4995/wrs.2016.4359>
- Hasan, Y. W., Mejbil, R. K., Konca, Y., Salman, G. Y., Najim, Y. S., & Khan, R. U. (2025). Effects of *Azolla pinnata* as a partial soybean meal substitute on growth, carcass traits, haemato-biochemical parameters, and tissue health of common carp (*Cyprinus carpio*). *Pakistan Veterinary Journal*, 45(4), 2059-2066. <https://doi.org/10.29261/pakvetj/2025.309>
- Hayat, M. N., Kumar, P., & Sazili, A. Q. (2023). Are spiritual, ethical, and eating qualities of poultry meat influenced by current and frequency during electrical water bath stunning? *Poultry Science*, 102(9), Article 102838. <https://doi.org/10.1016/j.psj.2023.102838>

- Idris, N. H., Muhammad, N. S., Abas, A., & Kasmn2, M. N. (2023). Are Malaysians ready for rabbit meat? An exploratory to assess readiness of Malaysians to accept rabbit meat as an alternative protein source. *Environment-Behaviour Proceedings Journal*, 8(SI15), 149-154. <https://doi.org/10.21834/e-bpj.v8iSI15.5099>
- Ikyume, T. T., Ogu, I. E., Okwori, I. A., & Shaahu, D. T. (2019). Growth performance and apparent nutrient digestibility of grower rabbits fed combinations of concentrate with grass and/or legume forage. *Journal of Multidisciplinary Research and Reviews*, 1(1), 41-45.
- Khatoon, A., Abidin, Z. U., Ali, A., Saleemi, M. K., Abbas, R. Z., Ijaz, M. U., Rehman, M. U., & Murtaza, B. (2025). Managing mycotoxins in animal/poultry feed through innovative control strategies: A review. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, 31(5), 581-602. <https://doi.org/10.9775/kvfd.2025.34700>
- Kumar, P., Sharma, N., Narnoliya, L. K., Verma, A. K., Umaraw, P., Mehta, N., Ismail-Fitry, M. R., Kaka, U., Yong-Meng, G., Lee, S. -J., & Sazili, A. Q. (2024). Improving quality and consumer acceptance of rabbit meat: Prospects and challenges. *Meat Science*, 219, Article 109660. <https://doi.org/10.1016/j.meatsci.2024.109660>
- Lamidi, W. A. & Akilapa, T. P. (2013). Performance characteristics of rabbits fed with *Leucaena* leaf meal based diet in natural housing system. *Agricultural Science Research Journal*, 3(5), 144-151.
- Lebas, F. (2004). *Reflections on rabbit nutrition with a special emphasis on feed ingredients utilisation* [Paper presentation]. 8th World Rabbit Congress, Puebla, Mexico.
- Mahanani, M. M. P., Kurniawati, A., Hanim, C., Anas, M. A., & Yusiati, L. M. (2020). Effect of (*Leucaena leucocephala*) leaves as tannin source on rumen microbial enzyme activities and in vitro gas production kinetics. *IOP Conference Series: Earth and Environmental Science*, 478, Article 012088. <https://doi.org/10.1088/1755-1315/478/1/012088>
- Makinde, O. J. (2016). Growth performance, carcass yield and blood profiles of growing rabbits fed concentrate diet supplemented with white lead tree (*Leucaena leucocephala*) or siratro (*Macroptilium atropurpureum*) leaves in North Central Nigeria. *Trakia Journal of Sciences*, 14(1), 80-86. <https://doi.org/10.15547/tjs.2016.01.011>
- Makinde, O. J., Ibe, E. A., & Ajibade, A. J. (2015). Response of growing rabbits to concentrate diet supplemented with *Leucaena* (*Leucaena leucocephala*) or siratro (*Macroptilium atropurpureum*) leaves. *Journal of Biology, Agriculture and Healthcare*, 5(12), 17-21.
- Makkar, H. P. S. (2005). In vitro gas methods for evaluation of feeds containing phytochemicals. *Animal Feed Science and Technology*, 123-124(Part 1), 291-302. <https://doi.org/10.1016/j.anifeedsci.2005.06.003>
- Mohammed, R. S., El Souda, S. S., Taie, H. A. A., Moharam, M. E., & Shaker, K. H. (2015). Antioxidant, antimicrobial activities of flavonoids glycoside from *Leucaena leucocephala* leaves. *Journal of Applied Pharmaceutical Science*, 5(6), 138-147. <https://doi.org/10.7324/JAPS.2015.50623>
- Mohd Aminuddin, Z., Hashim, R., Idris, S. H., Saidon, J., & Prihandono, I. (2023). Marketing issues in the cuniculture industry: The case of rabbit farmers in Hilir Perak, Malaysia. *Environment-Behaviour Proceedings Journal*, 8(23), 31-36. <https://doi.org/10.21834/ebpj.v8i23.4475>
- Montoya-Flores, M. D., Molina-Botero, I. C., Arango, J., Romano-Muñoz, J. L., Solorio-Sánchez, F. J., Aguilar-Pérez, C. F., & Ku-Vera, J. C. (2020). Effect of dried leaves of *Leucaena leucocephala* on rumen

- fermentation, rumen microbial population, and enteric methane production in crossbred heifers. *Animals*, 10(2), Article 300. <https://doi.org/10.3390/ani10020300>
- Mtenga, L. A., & Laswai, G. D. (1994). *Leucaena leucocephala* as feed for rabbits and pigs: Detailed chemical composition and effect of level of inclusion on performance. *Forest Ecology and Management*, 64(2-3), 249-257. [https://doi.org/10.1016/0378-1127\(94\)90299-2](https://doi.org/10.1016/0378-1127(94)90299-2)
- Nakamane, G., Harrison, S., Janthibordee, K., Srisomporn, W., & Phaikaew, C. (2019). Potential of *Leucaena* spp. as a feed resource for ruminant animals in Thailand. *Tropical Grasslands-Forrajeras Tropicales*, 7(4), 449-454. [https://doi.org/10.17138/tgft\(7\)449-454](https://doi.org/10.17138/tgft(7)449-454)
- Naqvi, S. Z. H., Maqbool, B., Arshad, M.I., Aderibigbe, A., and Gul, S.T. (2025). Food security and livestock: A comprehensive review of sustainability, challenges and innovative solutions. *Agrobiological Records*, 20, 50-58. <https://doi.org/10.47278/journal.abr/2025.019>
- Nguyen, B. C. Q., & Tawata, S. (2016). The chemistry and biological activities of mimosine: A review. *Phytotherapy Research*, 30(8), 1230-1242. <https://doi.org/10.1002/ptr.5636>
- Nursiwi, A., Ishartani, D., Sari, A. M., & Nisyah, K. (2018). Study on *Leucaena leucocephala* seed during fermentation: Sensory characteristic and changes on anti nutritional compounds and mimosine level. *IOP Conference Series: Earth and Environmental Science*, 102(1), Article 012093. <https://doi.org/10.1088/1755-1315/102/1/012093>
- Pal, A., Sharma, R. K., Kumar, R., & Barman, K. (2010). Effect of replacement of concentrate mixture with isonitrogenous leaf meal mixture on growth, nutrient utilisation, and rumen fermentation in goats. *Small Ruminant Research*, 91(2-3), 132-140. <https://doi.org/10.1016/j.smallrumres.2010.02.012>
- Pérez-Bautista, J. J., Candelaria-Martínez, B., Martínez-Martínez, R., Vicente-Pérez, R., Marcelo-Hernández, S., Herrera-Pérez, J., Grifaldo- Alcántara P. F., & Gómez-Vázquez, A. (2023). Productive behaviour of rabbits fed with *Leucaena leucocephala* (Lam.) de Wit, foliage in the fattening phase. *Agro Productividad*, 16(12), 89-93. <https://doi.org/10.32854/agrop.v16i12.2773>
- Rosida, D. F., Djajati, S., Nilamayu, Z. A., & Rosida. (2017). Antibacterial activity of *Leucaena leucocephala* extracts on growth of *Escherichia coli*. *Advanced Science Letters*, 23(12), 12268-12271. <https://doi.org/10.1166/asl.2017.10618>
- Safwat, A. M., Sarmiento-Franco, L., Santos-Ricalde, R. H., Nieves, D., & Sandoval-Castro, C. A. (2015). Estimating apparent nutrient digestibility of diets containing *Leucaena leucocephala* or *Moringa oleifera* leaf meals for growing rabbits by two methods. *Asian-Australasian Journal of Animal Sciences*, 28(8), 1155-1162. <https://doi.org/10.5713/ajas.14.0429>
- Safwat, A. M., Sarmiento-Franco, L., Santos-Ricalde, R., & Nieves, D. (2014). Effect of dietary inclusion of *Leucaena leucocephala* or *Moringa oleifera* leaf meal on performance of growing rabbits. *Tropical Animal Health and Production*, 46, 1193-1198. <https://doi.org/10.1007/s11250-014-0626-1>
- Sarkar, S., Mohini, M., Sharma, A., Tariq, H., & Pal, R. P. (2021). Effect of supplementing *Leucaena leucocephala* leaves alone or in conjunction with malic acid on nutrient utilisation, performance traits, and enteric methane emission in crossbred calves under tropical conditions. *Tropical Animal Health and Production*, 53, Article 514. <https://doi.org/10.1007/s11250-021-02941-7>

- Schofield, P., Mbugua, D. M., & Pell, A. N. (2001). Analysis of condensed tannins: A review. *Animal Feed Science and Technology*, 91(1-2), 21-40. [https://doi.org/10.1016/S0377-8401\(01\)00228-0](https://doi.org/10.1016/S0377-8401(01)00228-0)
- Shakak, A. O., Salah, K. B. H., Ashkan, M. F., Bahshwan, S. M. A., Mogadem, A., Aljohani, S., Aljumaa, M. A., Alnasri, E. O., Alfaleh, A. A., Albalawi, M. A., Hajri, A. K., Al-Ghamdi, F. A., Alrayes, Z. R., & Zghab, I. (2025). Multifunctional biogenic selenium nanoparticles from *Citrus sinensis* peel: In vivo antioxidant, antiviral, antibacterial, anticancer and neuroprotective assessment in rat model. *Pakistan Veterinary Journal*, 45(4), 1559-1571. <https://doi.org/10.29261/pakvetj/2025.333>
- Shelton, H. M., & Brewbaker, J. L. (1994). *Leucaena leucocephala*—The most widely used forage tree legume. *Forage Tree Legumes in Tropical Agriculture*.
- Silva, R. R., Rodrigues, R. C., Rodrigues, M. M., Abdalla, A. L., da Silva Cabral, L., da Costa Araújo, D. L., Olivera-Viciedo, D., da Silva, E. C., de Moura Zanine, A., de Jesus Ferreira, D., Santos, F. N. d. S., Costa, K. A. d. P., Santos, A. M. d. P., Rocha, P. L. O., & Perazzo, A. F. (2024). Fermentation and nutritive value of pineapple stubble silage supplemented with *Leucaena* hay. *Agronomy*, 14(9), Article 2140. <https://doi.org/10.3390/agronomy14092140>
- Tangendjaja, B., Rahardjo, Y. C., & Lowry, J. B. (1990). *Leucaena* leaf meal in the diet of growing rabbits: Evaluation and effect of a low-mimosine treatment. *Animal Feed Science and Technology*, 29(1-2), 63-72. [https://doi.org/10.1016/0377-8401\(90\)90094-O](https://doi.org/10.1016/0377-8401(90)90094-O)
- Usman, M., Qureshi, A. S., Shahid, R. U., & Deebea, F. (2025). Impact of biochar and organic acid-enriched diet on growth performance and intestinal histomorphometry in Japanese quail (*Coturnix coturnix japonica*). *Pakistan Veterinary Journal*, 45(1), 352-359. <https://doi.org/10.29261/pakvetj/2025.127>
- Utami, M. M. D., & Akbar, A. (2025). Enhancing nutrient intake, egg production, and egg quality by fermented *Leucaena leucocephala* leaf meal in a diet of laying quail. *Veterinary World*, 18(1), 133-140. <https://doi.org/10.14202/vetworld.2025.133-140>
- López Valoy, B., Cisneros López, M., Valdivié Navarro, M., Sotto Agüero, V., & Savón Valdés, L. (2012). Hidroforraje de *Leucaena leucocephala* para alimentar conejos [Hydro-forage of *Leucaena leucocephala* to feed rabbits]. *Revista de Producción Animal*, 24(1). <https://link.gale.com/apps/doc/A466297480/AONE?u=upml&sid=googleScholar&xid=72488453>
- Varga, M. (2014). Rabbit basic science. In *Textbook of Rabbit Medicine*, (2nd ed., pp. 3-108). <https://doi.org/10.1016/B978-0-7020-4979-8.00001-7>
- Verma, S. (2016). A review study on *Leucaena leucocephala*: A multipurpose tree. *International Journal of Scientific Research in Science and Technology*, 2(2), 103-105.
- Vijayakumar, S., & Srinivasan, P. (2018). Clinical management of spontaneous *Leucaena leucocephala* (Subabul) poisoning in non descriptive goat. *International Journal of Current Microbiology and Applied Sciences*, 7(10), 1148-1151. <https://doi.org/10.20546/ijcmas.2018.710.127>
- Wiratmini, N. I., Setyawati, I., Warmadewi, D. A., & Setiasih, N. L. E. (2021). Water-soaked *Leucaena leucocephala* leaf meal as feed for rabbits: Effect of inclusion on the performance. *IOP Conference Series: Earth and Environmental Science*, 709, Article 012058. <https://doi.org/10.1088/1755-1315/709/1/012058>
- Xuan, T. D., Elzaawely, A. A., Deba, F., Fukuta, M., & Tawata, S. (2006). Mimosine in *Leucaena* as a potent bio-herbicide. *Agronomy for Sustainable Development*, 26(2), 89-97. <https://doi.org/10.1051/agro:2006001>

- Zarina, Z., Ghazali, C. M. R., & Sam, S. T. (2017). Characterisation analysis for leaves of *Leucaena leucocephala* by using phytochemical screening assay. *AIP Conference Proceedings*, 1885(1), Article 020260. <https://doi.org/10.1063/1.5002454>
- Zayed, M. Z., Sallam, S. M. A., & Shetta, N. D. (2018). Review article on *Leucaena leucocephala* as one of the miracle timber trees. *International Journal of Pharmacy and Pharmaceutical Sciences*, 10(1), 1-7. <https://doi.org/10.22159/ijpps.2018v10i1.18250>

Field Phenotyping of Seven Malaysian Coconut Varieties for Yield Traits

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ABSTRACT

Although the coconut (*Cocos nucifera* L.) is of economic importance, the industry poses challenges of low productivity and limited seedling availability. This study utilised field phenotyping to evaluate the yield characteristics of four tall and three dwarf coconut varieties across three distinct locations in the West Coast regions of Peninsular Malaysia. A total of 210 samples, representing seven varieties, were collected using a nested design methodology. The analysis of the mean comparison between Malayan Yellow Dwarf (MYD) and Malayan Red Dwarf (MRD) varieties reveals that MYD exhibits a smaller leaf area but a faster maturation rate, whereas MRD demonstrates a greater leaf area and undergoes a slower maturation process. A yield trait comparison between Pandan and Tagnanan (TGG) shows Pandan having a significantly higher nut yield, while both varieties exhibit a similar maturation rate. Additionally, the mean comparison of yield traits of Malayan Tall (MT), Ceylon Tall (CT), and Rennell Tall (RT) indicates that all varieties have similar rates of maturation and average nut yield per year, but different values of Standing Stem Dry Weight (SSDW) and

Projected Leaf Area (PLA). Notably, RT exhibits a unique trait of having the lowest PLA among the three varieties. The correlation analysis identifies SSDW as the key factor for average nut yield per year in MRD, Pandan, and RT, as well as for maturation rate in MYD, MRD, and CT. In summary, the findings of this study can be leveraged for informed decision-making and strategic cultivation practices in the Malaysian coconut industry.

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INTRODUCTION

Coconut (*Cocos nucifera* L.) is a monocotyledonous perennial palm that belongs to the sub-family Cocoideae, which includes 27 genera and 600 species (Teulat et al. 2000). It is the only species within the genus *Cocos* and has 32 chromosomes ($2n = 16$) (Khairun et al., 2016). The primary taxonomic classification of coconut presents two varieties: tall and dwarf. The tall variety is characterised by cross-pollination, heterozygosity, a long lifespan, and slow maturation. Conversely, the dwarf variety is characterised by self-pollination, homozygosity, and a faster maturation rate than the tall variety (Perera et al., 2010). Dwarfs are typically found in close proximity to human settlements and display traits that are reflective of human selection, such as slow trunk growth and self-pollination (Alrifai & Marcone, 2019). In contrast, tall varieties demonstrate a wild-type phenotype with high phenotypic expression for all metrical traits and lack self-pollination (Raveendra et al., 2010). Given its multifaceted uses and the potential for human utilisation of every part of the plant, the coconut is commonly dubbed the "tree of life" (Burkill et al., 1966).

Coconut plays an important role in the agricultural landscape of Malaysia as it is ranked the fourth most important crop in terms of area planted after oil palm, rubber, and paddy (Yun, 2019). In 2022, coconut cultivation in Malaysia covered an estimated 84,935.9 hectares, with approximately 64,837 farmers engaged in its production. The coconut productivity in this reported year was 8.3 tan/ha, resulting in a total production of 604,428.4 metric tons (Ministry of Agriculture and Food Security, 2023). Coconut breeding has traditionally focused on the attainment of high nut yield as its primary objective, with subsequent priorities including precocity, reduced plant height, and improved resistance to biotic and abiotic stresses (Perera, 2012).

Malaysia has significant potential to increase its coconut productivity. This is because a large proportion of the existing coconut trees are senile and no longer productive (Abu Dardak & Mohd Yon, 2021). So, an overall increase in coconut yield is possible by replacing these trees with younger and more productive ones. Additionally, some coconut varieties have inherently low yield potential (Sivakumar et al., 2020). If farmers lack access to higher-yielding varieties, the consequences would be an overall reduction in coconut productivity. Planning for a large-scale replanting program with elite varieties with desirable traits, such as higher yield, becomes a distant goal because of the limited availability of quality seedlings. The limited availability of quality seedlings further hinders farmers' ability to replant senile trees with more productive ones. These issues highlight the need to address the challenges facing coconut farming in Malaysia. Therefore, expanding replanting initiatives is essential for revitalising the coconut sector and meeting current market demands. However, achieving this on a large scale remains a challenging goal.

This study highlights critical aspects of coconut germplasm conservation and utilisation. Exploration of yield traits in Malaysian coconut germplasm materials is a way to identify and safeguard genetic diversity within the Malaysian coconut gene pool.

Besides this, the current study aims to identify high-yielding coconut germplasm materials that can be applied in breeding programs, contributing to increased coconut production. Identifying high-yielding coconut germplasm materials can improve farming practices by allowing more coconuts to be produced on less land, which also contributes to sustainable agriculture. In the future, the use of data analytics, especially linear regression, may help develop predictive models that enhance research outcomes and estimate productivity more accurately.

This study systematically examines phenotypic variations in yield traits among seven selected Malaysian coconut germplasm materials, which include four tall varieties: Malayan Tall (MT), Rennell Tall (RT), Ceylon Tall (CT), and Tagnanan Tall (TGG), as well as three dwarf varieties: Pandan, Malayan Yellow Dwarf (MYD), and Malayan Red Dwarf (MRD). While past studies have primarily focused on molecular markers or limited field trials, our study offers a more thorough evaluation of yield traits through field phenotyping. Using this method, we gained a clearer understanding of germplasm potential, which can guide breeding efforts to develop higher-yielding coconut varieties. Current research marks the first comprehensive morphological assessment of coconut germplasm within the Department of Agriculture of Malaysia's repository, which was evaluated through a newly developed observational pipeline. Utilising unmanned aerial vehicles (UAVs) and cutting-edge open-source software, we collected and analysed reflectance data to estimate leaf area. These approaches provide a foundation for integrating advanced phenotyping techniques and tools into coconut research programs.

MATERIALS AND METHODS

Selection of Coconut Palms

A total of seven plots were identified within three agricultural centres under the Department of Agriculture of Malaysia (DOA), focusing on the data collection of seven coconut varieties: Malayan Red Dwarf (MRD) and Malayan Yellow Dwarf (MYD) at Agriculture Centre Lekir, Perak; Pandan and Tagnanan (TGG) at Agriculture Centre Teluk Bharu, Perak; as well as Malayan Tall (MT), Ceylon Tall (CT), and Rennal Tall (RT) at Agriculture Centre Parit Botak, Johor. Varietal comparisons were conducted only among palms grown under the same site and management conditions within each location (MRD and MYD at Lekir-Pandan; Pandan and TGG at Teluk Bharu; MT/CT/RT at Parit Botak). We did not compare varieties across sites. A total of 210 samples, comprising seven varieties, each with 30 nested samples, were selected, utilising a nested design approach. $n = 30$ per variety follows standard coconut germplasm evaluation (Santos et al., 1996) and was the maximum feasible due to limited uniform mature palms and labour-intensive phenotyping. This provided power to detect large differences (e.g., nut yield Pandan vs. TGG), though larger n would refine intravarietal variation estimates.

Palms were from the same germplasm block and planting year according to the DOA database. Physiological age was verified by trunk height and length of 11 leaf scars, and atypical or stressed palms were excluded. It should be noted that location effects, particularly when comparing tall versus dwarf varieties, cannot be fully separated from varietal effects. However, soil physicochemical properties were not measured at each site. Unmeasured soil variability may contribute to variation in SSDW and nut yield that is not due to genetics. Future studies should incorporate soil analysis, which clarifies how much of the variation is due to environmental factors.

Determination of Standing Stem Dry Weight (SSDW)

A non-destructive approach was used to determine the dry weight of standing stems. The stems' vertical height was measured from the ground to the base beneath the crown. For taller palms exceeding 3 meters, an unmanned aerial vehicle (UAV) model SJRC F11 4K Pro was employed for height measurement. To ensure reliable measurements, several validation steps were conducted. First, ground measurements were manually taken for palms shorter than 3 m to cross-check the UAV estimates. Besides this, UAV flights followed a consistent protocol, such as the camera being aligned parallel to the stem and flown vertically to the crown base, with GPS altitude hold and a gimbal used to reduce positional error. Finally, measurements were repeated across flights to check for consistency. It should be noted that formal statistical validation, such as regression between UAV and ground measurements, was not performed. Future studies should include systematic ground-truth calibration across different height classes.

The stem circumference was recorded at two specific points, namely 20 cm and 1.5 m above ground level, to obtain these measurements. A measuring tape was carefully wrapped around the stems of 30 selected palm trees from each variety. The calculation for SSDW was performed according to a method developed by Naresh et al., (2008), as shown in Equation 1:

$$\text{SSDW (kg)} = \text{Length (in m)} * \text{girth}^2 \text{ (in m, at 1.5 m above ground level)} * 41.14142 \quad [1]$$

The coefficient 41.14142 is pertinent for palm trees taller than 1.5 meters. For palms below 1.5 meters, a correction factor (F) was computed and employed in the SSDW calculation for dwarf palm trees, as outlined in Equation 2:

$$\text{SSDW (kg)} = \text{Length (in m)} * \text{girth}^2 \text{ (in m, at 20 cm above ground level)} * (H/1.5) * 41.14142 \quad [2]$$

where, H = Individual stem height

The correction factor considers the differences in measurement height (20 cm above ground) and scales the girth measurement to what it would have been at 1.5 meters. By

including the correction factor, the height difference was addressed, and the formula was made applicable to shorter palm trees.

Determination of the Length of 11 Leaf Scars

The length of 11 leaf scars was measured by recording the distance between the bottom of scar number 1 (above ground) and the bottom of scar number 11 using a measuring tape (Santos et al., 1996).

Determination of the Estimated Number of Fruits Per Bunch

The number of fruits per bunch was estimated by selecting three of the most mature bunches and visually counting the fruits with the eye from the ground. The recorded values were used to calculate the average number of fruits per bunch. This process of fruit estimation was repeated over six consecutive readings, taken at bimonthly intervals for dwarf, and monthly intervals for tall (Santos et al., 1996). The average number of fruits per bunch was recorded for each reading.

The yearly average was determined using a customised calculation method of Santos et al. (1996), specifically tailored to align with the research objectives and accommodate resource constraints as stated below in Equations 3 and 4:

$$\text{Tall: Estimated Number of Fruit per Bunch year-1} = \Sigma ((\bar{y}_1 + \bar{y}_2 + \bar{y}_3 + \bar{y}_4 + \bar{y}_5 + \bar{y}_6) / 6) * 12 \quad [3]$$

$$\text{Dwarf: Estimated Number of Fruit per Bunch year-1} = \Sigma ((\bar{y}_1 + \bar{y}_2 + \bar{y}_3 + \bar{y}_4 + \bar{y}_5 + \bar{y}_6) / 6) * 2 * 12 \quad [4]$$

where, $\bar{y}_i$ is the individual average yield.

Determination of Total Leaf Area

Leaf area (crown) of each palm was determined by employing image data acquisition and analysis through the utilisation of Plant Screen Mobile (PSM) (Müller-Linow et al., 2019), a smartphone application designed to estimate proxies of leaf area in diverse imaging situations, including field conditions. Image acquisition for tall palm trees was conducted using the UAV model SJRC F11 4K Pro, while images for dwarf palm trees were captured using smartphones, as the use of drones was not practical. In both scenarios, smartphones with identical camera calibrations were employed. Image post-processing was conducted for leaf area proxy estimation via the PSM app (Figure 1). The processed images were transformed into a CSV file by the PSM application, facilitating the extraction of the Projected Leaf Area (PLA) for each individual sample.

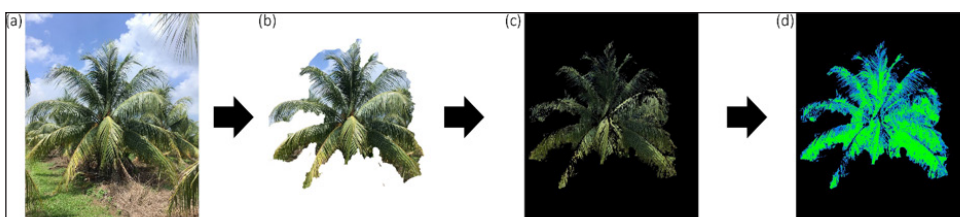


Figure 1. Steps of image post-processing and analysis using the Plant Screen Mobile App. (a) An image of sampled palms was captured, primarily focused on the crown. (b) AI-incorporated tools were employed to achieve precise extraction of specific sections within images. (c) The Plant Screen Mobile app used image masking in image processing to selectively conceal or reveal specific regions based on a selected greenness segmentation algorithm for precise manipulation and isolation of areas of interest in the image. (d) Plant Screen Mobile facilitated trait analysis and the development of proxies for total leaf area measured in pixels per square millimetre

Statistical Analysis

The Shapiro-Wilk test was performed for each set of yield trait data to assess its normality. In instances where datasets exhibited adherence to normal distribution, an independent samples t-test was employed to investigate significant differences between the means of two independent groups. Similarly, the one-way analysis of variance (ANOVA) was employed to assess the impact of multiple factors on the observed data when three factors were under analysis. Subsequently, post hoc testing was conducted using the Tukey Honestly Significant Difference (HSD) test to compare means, depending upon the detection of significant differences in the ANOVA. If the data for the yield trait did not show a normal distribution, non-parametric tests were employed. The Mann-Whitney U test was utilised to ascertain significant differences in cases where comparison was limited to two varieties. For analyses involving more than two varieties, the Kruskal-Wallis test is used for comparison. Afterwards, post hoc comparisons were performed using the Dunn test to examine differences in mean ranks whenever the Kruskal-Wallis test indicated significant variation. Data was analysed in two stages. First, an overall test (ANOVA or Kruskal-Wallis) was conducted for each trait. When significant differences were observed, post hoc pairwise comparisons were carried out using Tukey HSD for ANOVA and the Dunn test for Kruskal-Wallis to control family-wise error. No additional corrections were applied across traits, as each trait represents a distinct biological outcome. Sample sizes were balanced, with 30 individuals per variety at each site, providing sufficient power to detect moderate to large differences. Some comparisons, such as nut yield among MT, CT, and RT varieties, were not significant, possibly due to variance limiting detectability. Confidence intervals were not reported in this study but would help quantify estimation precision beyond p-values. Future analyses will include confidence intervals alongside mean comparisons and statistical tests.

RESULTS AND DISCUSSION

The Shapiro-Wilk test confirmed that the MYD and MRD varieties grown at Agriculture Centre Lekir, Perak, exhibited normal distribution for all yield traits, except the length of 11 leaf scars ($p < 0.05$) (Table 1). Therefore, an independent t-test was applied for the analysis, except for the length of 11 leaf scars, which underwent a Mann-Whitney U test

Table 1
Normality assumption of the yield trait data of seven coconut varieties at three different locations using the Shapiro-Wilk test

Yield Trait	Location	Variety	Shapiro-Wilk Test Statistic (W)	p-value	Interpretation of Normality
Average nut yield per year	Agriculture Centre Lekir, Perak	MYD	0.95	0.16	Normal
		MRD	0.95	0.15	Normal
	Agriculture Centre Teluk Bharu, Perak	Pandan	0.91	0.01	Not normal
		TGG	0.94	0.08	Normal
	Agriculture Centre Teluk Bharu, Johor	MT	0.96	0.25	Normal
		CT	0.93	0.06	Normal
RT		0.95	0.14	Normal	
Standing stem dry weight	Agriculture Centre Lekir, Perak	MYD	0.96	0.32	Normal
		MRD	0.93	0.06	Normal
	Agriculture Centre Teluk Bharu, Perak	Pandan	0.99	0.94	Normal
		TGG	0.68	0.00	Not normal
	Agriculture Centre Teluk Bharu, Johor	MT	0.94	0.12	Normal
		CT	0.97	0.57	Normal
RT		0.95	0.18	Normal	
Projected leaf area	Agriculture Centre Lekir, Perak	MYD	0.97	0.49	Normal
		MRD	0.99	0.96	Normal
	Agriculture Centre Teluk Bharu, Perak	Pandan	0.96	0.94	Normal
		TGG	0.81	0.00	Not normal
	Agriculture Centre Teluk Bharu, Johor	MT	0.87	0.00	Not normal
		CT	0.78	0.00	Not normal
RT		0.69	0.00	Not normal	
Length of 11 scars	Agriculture Centre Lekir, Perak	MYD	0.94	0.09	Normal
		MRD	0.85	0.00	Not normal
	Agriculture Centre Teluk Bharu, Perak	Pandan	0.91	0.02	Not normal
		TGG	0.96	0.23	Normal
	Agriculture Centre Teluk Bharu, Johor	MT	0.95	0.57	Normal
		CT	0.94	0.11	Normal
RT		0.96	0.30	Normal	

Note. MYD = Malayan Yellow Dwarf, MRD = Malayan Red Dwarf, TGG = Tagnanan, MT = Malayan Tall, CT = Ceylon Tall, RT = Rennell Tall

(Figure 2). For average nut yield per year and SSDW, the independent t-test did not reveal significant differences between the two varieties ($p = 0.87$ and $p = 0.20$, respectively, both $p > 0.05$), indicating that the yields of both varieties were similar. However, for PLA, the independent t-test indicated a statistically significant difference ($p = 0.04$, $p < 0.05$), suggesting different performance between MYD and MRD varieties in this regard. Specifically, the MRD exhibited significantly higher values in both PLA and the length of 11 leaf scars as compared to MYD. These findings suggest that leaf growth does not significantly impact the storage of dry matter and soluble carbohydrates in the stem for both varieties examined. These results are consistent with prior research that suggests a reduction in leaf area does not exert a significant effect on the carbohydrate reserves stored in the stem (Mialet-Serra et al. 2008). In addition, palms with larger leaf areas in the MRD group demonstrated a slower rate of maturation, while palms with smaller leaf areas in the MYD group showed a faster maturation rate. The limited existing literature on the relationship between fast maturation and leaf area in dwarf varieties suggests that these findings could provide preliminary insights into how to accelerate maturity in both varieties. Based on the available information, it can be inferred that MYD reaches maturity faster than MRD, with no significant difference in nut production between MRD and MYD. In the case of the length of 11 leaf scars, the Mann-Whitney U test demonstrated a highly significant difference ($p = 5.39 \times 10^{-6}$, $p < 0.05$) between the two varieties.

In contrast to MYD and MRD, the Shapiro-Wilk test indicated non-normal distributions ($p < 0.05$) across all examined yield traits in Pandan and TGG varieties at Agriculture Centre Teluk Bharu, Perak (Table 1). Therefore, the Mann-Whitney U test was used for further analysis, which revealed significant differences between the two varieties for

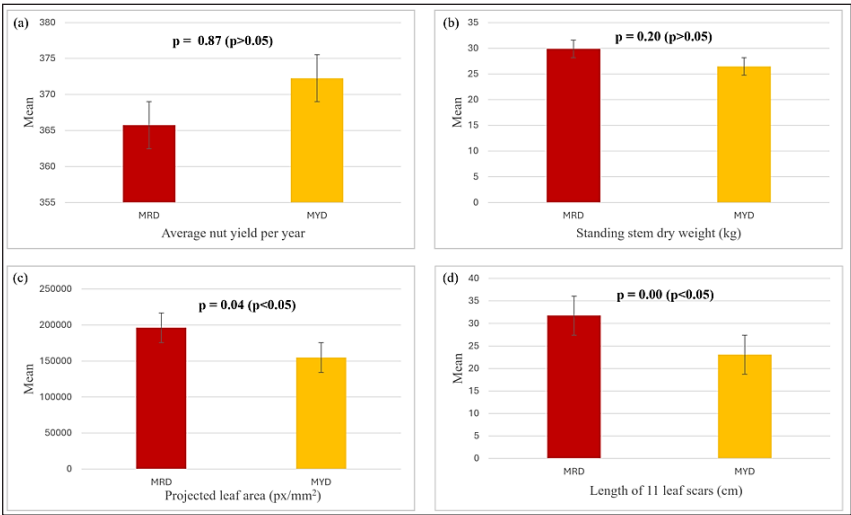


Figure 2. Comparative analysis of yield traits between Malayan Yellow Dwarf (MYD) and Malayan Red Dwarf (MRD) varieties

average nut yield per year ($p = 7.37 \times 10^{-11}$) and SSDW ($p = 1.21 \times 10^{-10}$) (Figure 3). Pandan demonstrates higher values in both aspects, indicating potential productivity advantages. Three Pandan palms, namely palm 01/0089, palm 01/0096, and palm 01/0103, yielded exceptionally high nut yields of 744, 720, and 708 nuts per palm per year, respectively. These yields represented increases of approximately 54%, 49%, and 46%, respectively, compared to the average nut yield per year of 483.6 nuts/palm/year for the Pandan variety. In contrast, palm 02/0057 of the TGG variety produced only 32 nuts per palm per year, compared with the variety’s average of 162.8 nuts per palm per year. Therefore, this palm showed approximately an 80.42% decrease in nut yield compared to the mean nut yield of the TGG variety. These results are consistent with earlier findings, which reported yield potential and yield gap ranging from 31% to 87% (Crisostomo et al. 2023). Expanding on the findings of prior research (Thomas et al. 2005), which identified dry matter production and efficient distribution as pivotal for coconut palm productivity, our study demonstrates Pandan's high nut yield despite markedly lower SSDW, contrasting with TGG's lower nut yield with high SSDW. This suggests that Pandan efficiently allocates energy to nut production, effectively converting stem-stored soluble sugars into yield.

As for PLA and the length of 11 leaf scars, no significant differences were observed, with a p-value of 0.44 and 0.09, respectively. Pandan, a variety that originated in Thailand as a spontaneous mutation of the green dwarf type (Dumhai et al. 2019), demonstrates a maturation rate similar to that of TGG, a tall variety as observed in this study. This similarity in maturation rates may be attributed to its cultivation in regions far from its native origin. The maturation rate of Pandan outside its place of origin has not been documented in previous research, highlighting a gap in existing studies.

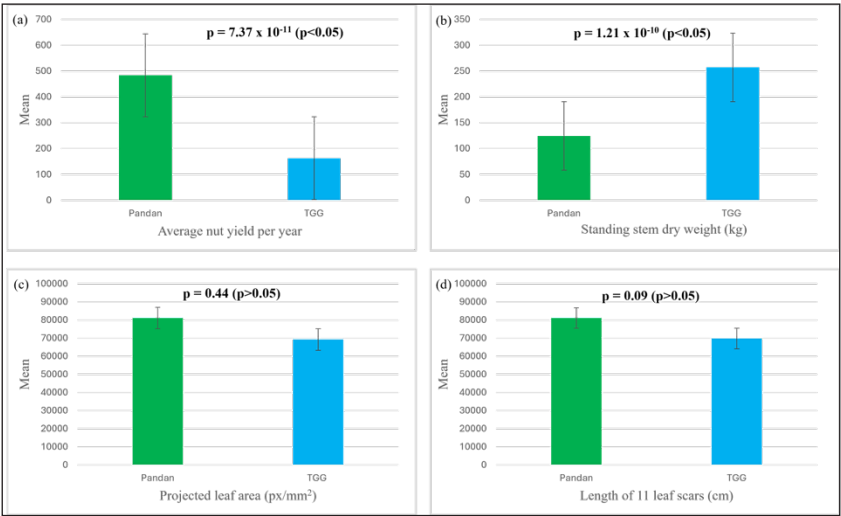


Figure 3. Comparative analysis of yield traits between Pandan and Tagnanan (TGG) varieties

Next, since the datasets of MT, CT, and RT varieties grown at the Agriculture Centre, Teluk Bharu, Johor showed normal distributions across all yield traits ($p > 0.05$), except for PLA ($p < 0.05$) (Table 1), ANOVA was utilised for statistical analysis. In the case of PLA, the Kruskal-Wallis test was employed. The ANOVA test conducted on the average nut yield per year of MT, CT, and RT varieties yielded a p-value of 0.04 ($p < 0.05$), indicating a significant difference among the groups (Figure 4). Similarly, for the SSDW of MT, CT, and RT varieties, the ANOVA test resulted in a p-value of 6.44×10^{-11} ($p < 0.05$), indicating a significant difference between the groups. However, for the length of 11 leaf scars, the ANOVA test produced a p-value of 0.80 ($p > 0.05$), indicating no significant difference among the groups. In contrast, the Kruskal-Wallis test conducted on the varieties MT, CT, and RT for the parameter PLA yielded a p-value of 6.06×10^{-4} ($p < 0.05$), indicating a significant difference among the groups.

Additional analysis was conducted through post hoc testing (Figure 5) on the average nut yield per year and SSDW, both employing Tukey's Honestly Significant Difference (HSD), and PLA with Dunn's test. This analysis was conducted to identify significant differences among groups, helping to draw more precise conclusions about the relationships between variables. The mean comparisons for average nut yield per year (Figure 5a) revealed differences of 4.80 between MT and CT, 43.67 between MT and RT, as well as 38.87 between CT and RT. These differences fall below the HSD 0.05 threshold of 44.45. Consequently, no significant differences were observed among the analysed pairs of MT-CT, MT-RT, and CT-RT at the 0.05 significance level, suggesting that the three varieties have comparable nut yields.

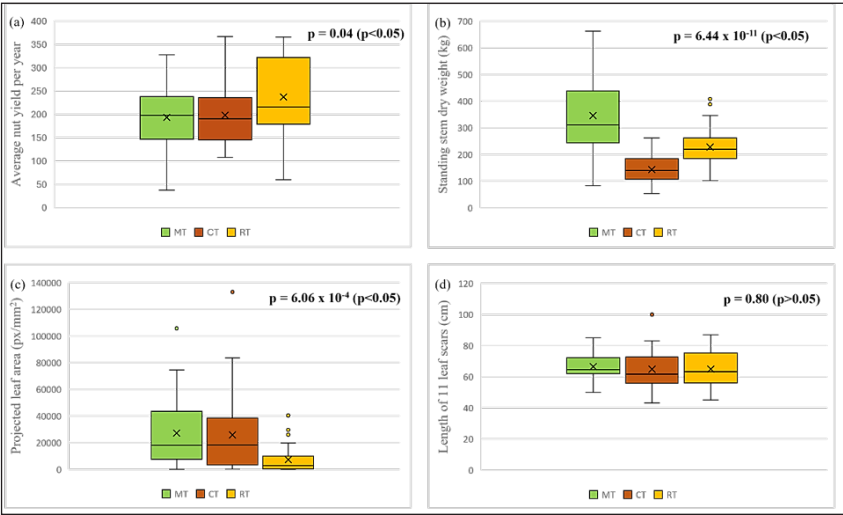


Figure 4. Comparative analysis of yield traits in Malayan Tall (MT), Ceylon Tall (CT), and Rennell Tall (RT) varieties

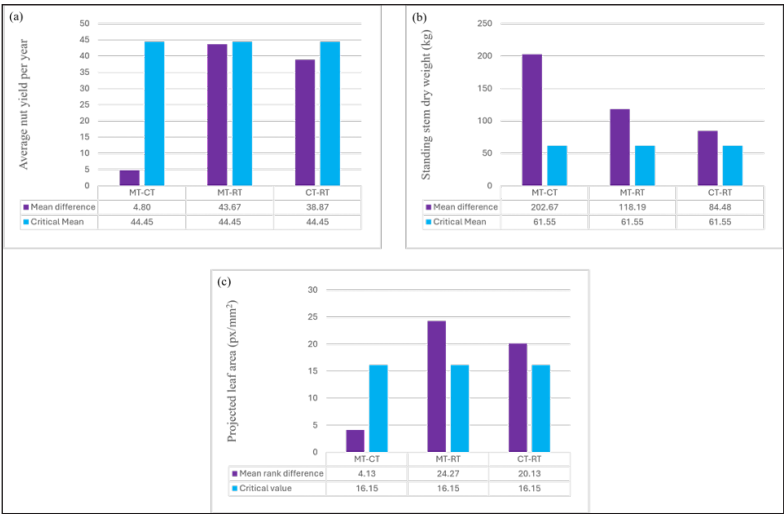


Figure 5. Comparisons of average nut yield per year, Standing Stem Dry Weight (SSDW), and Projected Leaf Area (PLA) among Malayan Tall (MT), Ceylon Tall (CT), and Rennell Tall (RT) varieties

In the comparison of mean SSDW among MT, CT, and RT varieties, all pairwise comparisons exhibited significant differences between their respective means (Figure 5b). The mean differences surpassed the critical value of HSD 0.05 at 61.56. Specifically, the mean differences for MT and CT, MT and RT, as well as CT and RT were 202.67, 118.19, and 84.47, respectively. The mean rank difference analysis for PLA across MT, CT, and RT varieties indicated significant differences in the mean ranks of the MT and RT pair (mean rank difference: 24.27 > critical value: 16.15) and the CT-RT pair (mean rank: 20.13 > critical value: 16.15) (Figure 5c). Conversely, no significant difference was observed in the MT-CT pair, with a mean rank difference of 4.13 < critical value: 16.15. The findings of the current study suggest that while there is variation in dry matter accumulation (SSDW) and PLA, the nut yield remains similar among the three tall palms, possibly due to shared characteristics such as soil nutrient extraction ability and comparable photosynthetic energy allocation toward nut yield. This variation in SSDW and PLA is aligned with previous research that suggests coconut, being less domesticated, still possesses characteristics inherited from its wild ancestors, leading to non-uniform vegetative growth (Foale, 2003). Nevertheless, the variation in the dry matter accumulation in the stem and leaf area among these three varieties suggests a consistent trend. However, these variations do not result in differences in nut yield among the three tall varieties.

The limitation of this study is that each variety was evaluated at a single location. This restricts how well the findings can be generalised across Malaysia's diverse agro-climatic zones and the range of management practices used on different farms. Therefore, multi-location trials are needed to better capture genotype × environment interactions and provide more robust recommendations for coconut varieties.

Correlation analyses were performed to investigate the dependency and potential influences among several growth and yield traits of coconut varieties. These traits include the average nut yield per year, SSDW, PLA, and the length of 11 leaf scars (Figure 6). Notably, the present investigation reveals a strong positive correlation between SSDW and the average nut yield per year in MRD, Pandan, and RT (Figure 6), suggesting a potential physiological link whereby higher stem dry matter accumulation may reflect greater carbohydrate storage available for reproductive growth. We cannot conclude that one factor causes another based on correlation alone. Future studies, such as path analysis or controlled experiments, are needed to better understand how stem dry matter accumulation influences nut yield efficiency. This correlation serves as an initial step in understanding the association between stem dry matter accumulation and its allocation towards nut production across the examined varieties. Previous literature suggests that the stem functions as a storage site for soluble sugars, particularly sucrose (Mialet-Serra et al., 2005). Carbohydrates are stored in the stem for medium-term storage, which predominantly benefits the plant during periods of increased energy demand, such as seed development or in response to drought-

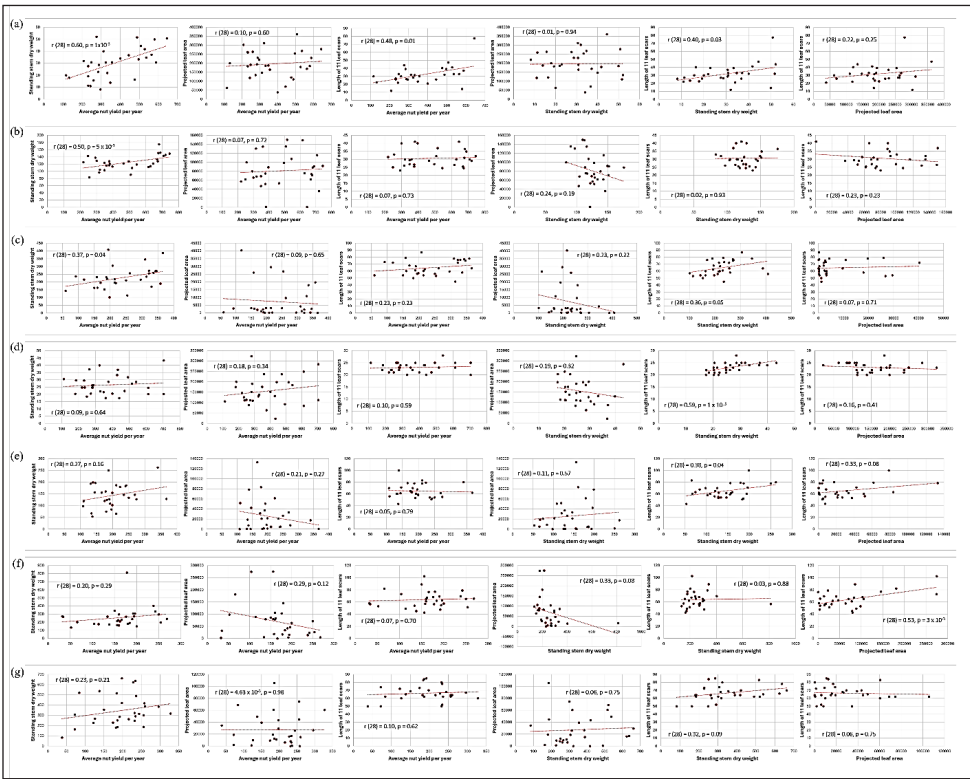


Figure 6. Correlation graphs illustrating the correlation coefficient between the yield traits of (a) Malayan Red Dwarf, (b) Pandan, (c) Rennel Tall, (d) Malayan Yellow Dwarf, (e) Ceylon Tall, (f) Tagnanan Tall, (g) Malayan Tall varieties

induced water stress (Kozłowski, 1992; Loescher et al., 1990; Oliveira & Priestley, 1988). In tropical regions where coconut trees thrive, the stored carbohydrate in the stem remains to play a significant role, despite relatively stable environmental conditions. Although coconut trees are known for their continual fruit production and ability to remain green throughout the year, it is vital to maintain carbohydrate reserves in order to support growth and fruiting activities (Mialet-Serra et al., 2005). However, there is a lack of studies on the relationship between the stem dry matter accumulation and the nut yield across specific varieties, thereby creating gaps in the knowledge of varietal-specific growth and fruiting dynamics. The present study has addressed this gap by identifying the specific variety which has the most efficient translation of stem dry matter to nut yield.

Next, the length of 11 leaf scars was examined to evaluate how the rate of maturation affects the overall growth and fruiting dynamics of each variety. These findings may facilitate the selection of palms that can produce fruit earlier and yield more nuts, supported by empirical evidence. Out of the seven varieties, only MRD demonstrates a noteworthy positive correlation between the length of 11 leaf scars and the average nut yield per year (Fig. 6). This implies that the slower maturation of MRD leads to an increase in the average nut yield per year, suggesting that the current study did not identify a variety that matures early and has a high yield. The length of 11 leaf scars is a key indicator in evaluating the growth pattern of coconut palms, indicating their precocious and early bearing nature (Mohamad Zaki et al., 2023). The initial inflorescence usually emerges in conjunction with the fronds, typically situated between the 10th and 12th frond positions (Foale, 2003). Previous studies, as well as the farmers' interests, were mostly focused on identifying coconut palms with short stems and early maturity that exhibit quick fruit-bearing capabilities exclusively (Novarianto et al., 2016). Although Bido Tall, a variety from Indonesia, has been described as having a short stem, faster maturation, and abundant yield compared to other tall varieties (Novarianto et al., 2016), there is little evidence to support its correlation with early bearing nature and yield potential. This study represents the first comprehensive investigation into the early bearing capacity in relation to nut yield.

Investigating the correlation between SSDW and the length of 11 leaf scars in the seven varieties of coconut palms in the present study holds promise for interpreting several factors of plant physiology and growth dynamics. This includes understanding nutrient partitioning, which refers to the distribution of soluble sugars in coconut palms that might explain the resource allocation within the plant. Examining the correlation with maturation rate could reveal whether faster-maturing palms use different strategies for sugar accumulation and distribution compared to slower-maturing palms. This study may help us understand how coconut palms cope with stress. The results of the present study reveal a positive correlation between SSDW and the length of 11 leaf scars in the MRD, MYD, and CT varieties (Figure 6). This suggests that in these three varieties, increased

accumulation of dry matter in the stem is associated with a slower maturation. Previous findings show that carbohydrate reserves function like signalling molecules, influencing various developmental and physiological processes within the plant (Mialet-Serra et al., 2005; Luquet et al., 2007). When plants have plenty of carbohydrate reserves, they often use these resources to grow more leaves or flowers. In contrast, if reserves are low, growth and maturation slow down until conditions improve and carbohydrates can be replenished. This phenomenon is referred to as sugar signalling (Roitsch et al., 2000; Rolland et al., 2002; Gupta & Kaur, 2005). Essentially, these carbohydrate stores facilitate plant adaptation to environmental fluctuations, serving as a form of resilience during challenging conditions (Wingler et al., 2006).

Examining the relationship between PLA and the length of 11 leaf scars provides a way to evaluate growth rate and understand how leaf size corresponds to scar length. This knowledge is crucial for understanding growth dynamics. Furthermore, the findings can be used for predictive modelling, where PLA's predictive capacity regarding scar length can inform models for forecasting maturation rates. The findings of the present study unveiled that only the TGG variety exhibits a significant positive correlation between PLA and the length of 11 leaf scars (Figure 6). This suggests that an augmentation in leaf area is associated with a deceleration of the process of maturation, specifically in the TGG variety. Consequently, it implies that a reduced efficacy in light interception during the transition from the juvenile phase to the productive stage may underline the observed phenomenon of slowed growth in TGG. Previous literature, as emphasised by Foale (2003), suggests that the crown shape has an impact on the efficiency of light interception. The current findings indicate that rapid leaf growth potentially impedes the optimal photosynthetic capacity necessary for TGG's rapid maturation. The slow growth of coconuts during the juvenile phase is attributed to decreased photosynthetic rates caused by the self-shading effect of upper canopy fronds (Corley, 1983). Consequently, compared to other varieties, TGG exhibits a slower transition from the juvenile to production phase due to the rapid growth of leaves. Based on the Pearson correlation analysis, MT is the only variety among the seven varieties that shows an insignificant, small positive correlation between the six yield parameters (Figure 6).

This study presents a novel pipeline for high-throughput coconut phenotyping using UAVs, mobile applications, and AI tools, representing the first application of these technologies in this field. A key challenge in coconut phenotyping is the labour-intensive and time-consuming estimation of total leaf area and stem height measurement, especially for the tall varieties. The Standardised Research Manual in Coconut Breeding (STANTECH) adopted a non-destructive method for estimating total leaf area, a technique that was previously widely used for estimating the leaf area of oil palm (Hardon et al., 1969). This method has been enhanced in this study by integrating novel tools and software into the data collection process. The PSM, an Android-based mobile app, has the potential

to make phenotyping coconut leafiness easier, faster, and more precise compared to the traditional methods (Müller-Linow et al., 2019). Integrating the PSM app with UAV technology is a novel approach for coconut phenotyping used in this study to capture and digitally measure proxies for the total leaf area of coconut palms, making measurements, especially for tall varieties, more accessible. Consequently, this pipeline for generating phenotyping data addresses the common bottleneck in coconut field phenotyping, enhancing data accuracy while reducing both time and cost associated with data collection (Arumugam & Md Hatta, 2022).

CONCLUSION

This study employs field phenotyping to evaluate yield traits in seven coconut varieties (four tall and three dwarf), sourced from DOA's germplasm collection. The findings indicate that MRD variety has a larger leaf area and a slower maturation rate compared to MYD, which exhibits a smaller leaf area but a faster maturation rate. Both MYD and MRD demonstrate similar nut yields. In MRD, yield traits are positively correlated with average nut yield per year, SSDW, and length of 11 leaf scars, while SSDW is positively correlated with the length of 11 leaf scars. In MYD, only SSDW is positively correlated with the length of 11 leaf scars. Pandan variety exhibits significantly higher nut yields compared to TGG, while TGG has a significantly higher SSDW value than Pandan. Pandan's exceptional nut yield, notably from palm number 01/0089, suggests that dry matter is effectively produced and allocated to support higher yield. Pandan and TGG show no significant differences in PLA and length of 11 leaf scars, indicating similar maturation rates despite their different statures.

Pandan's nut yield is positively related to SSDW, indicating effective production and distribution of dry matter. In TGG, PLA is positively correlated with the length of 11 leaf scars, suggesting faster maturation can be achieved with less leaf area without affecting nut yield. By comparing MT, CT, and RT, there are no distinctions in nut yield, but significant differences exist in SSDW and PLA. RT shows unique leaf development with the lowest means among the three varieties. The correlation analysis indicates that there is no association between yield traits in MT, while in RT, average nut yield per year is positively correlated with SSDW.

This study significantly advances coconut breeding programs by identifying high-yielding varieties and uncovering trait correlations. Future research will refine phenotyping strategies by integrating tools and technologies for high-throughput phenotyping, utilising innovative technologies for precise data collection and adapting to ongoing technological advancements. The outcomes lay a foundation for elucidating the molecular pathways and key genes underlying the coconut yield through the application of advanced omics technologies. It is crucial to select coconut palms with distinct phenotypes from the present study to perform future comprehensive investigations.

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DISCLAIMER

The authors confirm that no generative artificial intelligence (AI) tools were used in the writing, analysis, or preparation of this manuscript.

REFERENCES

- Abu Dardak, R., & Mohd Yon, R. (2021). *Strengthening the coconut industry in Malaysia*. Food and Fertiliser Technology Centre for the Asian and Pacific Region (FFTC-AP). <https://ap.ffc.org.tw/article/2938>
- Alrifai, O., & Marcone, M. F. (2019). Coconut - The tree of life - Improvement by biotechnology. In M. Moo-Young (Ed.), *Comprehensive Biotechnology* (3rd ed., Vol. 4, pp. 586-594). Pergamon. <https://doi.org/10.1016/B978-0-444-64046-8.00464-X>
- Arumugam, T., & Md Hatta, M. A. (2022). Improving coconut using modern breeding technologies: Challenges and opportunities. *Plants*, 11(24), Article 3414. <https://doi.org/10.3390/plants11243414>
- Burkill, I. H., Birtwistle, W., Foxworthy, F. W., Scrivenor, J. B., & Watson, J. G. (1966). *A dictionary of the economic products of the Malay Peninsula*. Governments of Malaysia and Singapore by the Ministry of Agriculture and Co-operatives.
- Corley, R. H. V. (1983). Potential productivity of tropical perennial crops. *Experimental Agriculture*, 19(3), 217-237. <https://doi.org/10.1017/S0014479700022742>
- Crisostomo, S. D., Cruz, C. D. V. D., Quilloy, R. B., & Reaño, C. E. (2023). Narrowing the yield gap of coconut (*Cocos nucifera* L.) through integrated nutrient management in the Philippines: An on-farm experiment approach. *IOP Conference Series: Earth and Environmental Science*, 1235(1), Article 012008. <https://doi.org/10.1088/1755-1315/1235/1/012008>
- Dumhai, R., Wanchana, S., Saensuk, C., Choowongkamon, K., Mahatheeranont, S., Kraithong, T., Toojinda, T., Vanavichit, A., & Arikrit, S. (2019). Discovery of a novel *CnAMADH2* allele associated with higher levels of 2-acetyl-1-pyrroline (2AP) in yellow dwarf coconut (*Cocos nucifera* L.). *Scientia Horticulturae*, 243, 490-497. <https://doi.org/10.1016/j.scienta.2018.09.005>
- Foale, M. (2003). *The coconut odyssey: The bounteous possibilities of the tree of life*. Australian Centre for International Agricultural Research.
- Gupta, A. K., & Kaur, N. (2005). Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Biosciences*, 30(5), 761-776. <https://doi.org/10.1007/BF02703574>
- Hardon, J. J., Williams, C. N., & Watson, I. (1969). Leaf area and yield in the oil palm in Malaya. *Experimental Agriculture*, 5(1), 25-32. <https://doi.org/10.1017/S0014479700009935>

- Khairun, H. N., Jaafar, M. A., Shahril, A. R., Sentoer, G. K., Siti, N., Fairuz, M. Y., Habibuddin, H., & Norzihan, A. (2016). Genetic diversity evaluation of MARDI's coconut (*Cocos nucifera* L.) germplasm using simple sequence repeats. *Cord*, 32(2), 37-45. <https://doi.org/10.37833/cord.v32i2.34>
- Kozlowski, T. T. (1992). Carbohydrate sources and sinks in woody plants. *The Botanical Review*, 58(2), 107-222. <https://doi.org/10.1007/BF02858600>
- Loescher, W., McCamant, T., & Keller, J. (1990). Carbohydrate reserves, translocation, and storage in woody plant roots. *HortScience*, 25(3), 274-281. <https://doi.org/10.21273/HORTSCI.25.3.274>
- Luquet, D., Song, Y. H., Elbelt, S., This, D., Clément-Vidal, A., Périn, C., Fabre, D., & Dingkuhn, M. (2007). Model-assisted physiological analysis of Phyllo, a rice architectural mutant. *Functional Plant Biology*, 34(1), 11-23. <https://doi.org/10.1071/FP06180>
- Mialet-Serra, I., Clément, A., Sonderegger, N., Rounsard, O., Jourdan, C., Labouisse, J., & Dingkuhn, M. (2005). Assimilate storage in vegetative organs of coconut (*Cocos nucifera*). *Experimental Agriculture*, 41(2), 161-174. <https://doi.org/10.1017/S0014479704002467>
- Mialet-Serra, I., Clement-Vidal, A., Rounsard, O., Jourdan, C., & Dingkuhn, M. (2008). Whole-plant adjustments in coconut (*Cocos nucifera*) in response to sink-source imbalance. *Tree Physiology*, 28(8), 1199-1209. <https://doi.org/10.1093/treephys/28.8.1199>
- Ministry of Agriculture and Food Security. (2023). *Malaysia agrofood in figures*. Ministry of Agriculture and Food Security.
- Mohamad Zaki, N. A., Kingimi, M., Naharudin, N. S., Jusoh, M., Ghazali, M. N., & Sinniah, U. R. (2023). Variability of fruit morphology, yield, and quality in the Tagnanan population as MATAG pollen source. *International Journal of Agriculture, Forestry and Plantation*, 13, 197-203.
- Müller-Linow, M., Wilhelm, J., Briesse, C., Wojciechowski, T., Schurr, U., & Fiorani, F. (2019). Plant Screen Mobile: An open-source mobile device app for plant trait analysis. *Plant Methods*, 15, Article 2. <https://doi.org/10.1186/s13007-019-0386-z>
- Naresh, K., Kasthuri, B., & George, J. (2008). A method for non-destructive estimation of dry weight of coconut stem. *Journal of Plantation Crops*, 36(3), 296-299.
- Novariato, H., Santosa, B., Tulalo, M., Mawardi, S., & Maskromo, I. (2016). Varietal improvement in coconut in Indonesia. In *Proceedings of the XLVII APCC Cocotech Conference & Exhibition*, Bali, Indonesia.
- Oliveira, C., & Priestley, C. (1988). Carbohydrate reserves in deciduous fruit trees. In J. Janick (Ed.), *Horticultural Reviews*, (pp. 403-430). Wiley Online Library. <https://doi.org/10.1002/9781118060834.ch10>
- Perera, L., Perera, S. A. C. N., Bandaranayake, C. K., & Harries, H. C. (2010). Coconut. In J. Vollmann & I. Rajcan (Eds.), *Oil Crops* (pp. 369-396). Springer. https://doi.org/10.1007/978-0-387-77594-4_12
- Perera, S. A. C. N. (2012). Coconut. In S. K. Gupta (Ed.), *Technological Innovations in Major World Oil Crops, Volume 1: Breeding* (pp. 201-218). Springer. https://doi.org/10.1007/978-1-4614-0356-2_8
- Raveendra, T. S., Ramanathan, T., Nallathambi, G., & Vijayaraghavan, H. (1987). Metroglyph analysis in coconut (*Cocos nucifera* L.). *Cocos: Journal of the Coconut Research Institute of Sri Lanka*, 5, 32-38. <https://doi.org/10.4038/cocos.v5i0.2048>

- Roitsch, T., Ehneß, R., Goetz, M., Hause, B., Hofmann, M., & Sinha, A. K. (2000). Regulation and function of extracellular invertase from higher plants in relation to assimilate partitioning, stress responses and sugar signalling. *Australian Journal of Plant Physiology*, 27, 815-825. <https://doi.org/10.1071/PP00001>
- Rolland, F., Moore, B., & Sheen, J. (2002). Sugar sensing and signalling in plants. *The Plant Cell*, 14(Suppl 1), S185-S205. <https://doi.org/10.1105/tpc.010455>
- Santos, G. A., Batugal, P. A., Othman, A., Baudouin, L., & Labouisse, J. (1996). *Manual on standardised research techniques in coconut breeding*. International Plant Genetic Resources Institute.
- Sivakumar, V., Subramanian, A., Geethanjali, S., Praneetha, S., & Maheswarappa, H. P. (2020). Assessment of genetic variability for growth, floral, yield and its component traits in coconut (*Cocos nucifera* L.). *Electronic Journal of Plant Breeding*, 11(3), 809-813. <https://doi.org/10.37992/2020.1103.133>
- Teulat, B., Aldam, C., Trehin, R., Lebrun, P., Barker, J. H. A., Arnold, G. M., Karp, A., Baudouin, L., & Rognon, F. (2000). An analysis of genetic diversity in coconut (*Cocos nucifera*) populations from across the geographic range using sequence-tagged microsatellites (SSRs) and AFLPs. *Theoretical and Applied Genetics*, 100(5), 764-771. <https://doi.org/10.1007/s001220051350>
- Thomas, S., Rajagopal, V., Kumar, S., Vadivel, A., & Cherian, V. (2005). Stability analysis of dry matter production and yield components of coconut in two agroclimatic regions of India. *Indian Journal of Plant Physiology*, 10(1), 101-108.
- Wingler, A., Purdy, S., MacLean, J. A., & Pourtau, N. (2006). The role of sugars in integrating environmental signals during the regulation of leaf senescence. *Journal of Experimental Botany*, 57(2), 391-399. <https://doi.org/10.1093/jxb/eri279>
- Yun, T. Z. (2019). Agriculture: A coconut revival. *The Edge Malaysia*. <https://theedgemalaysia.com/article/agriculture-coconut-revival>

A Direct Extraction Technique Enriched the Polyphenol Compositions and Their Biochemical Activities Obtained from *Garcinia* Species: An Evaluative Study

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ABSTRACT

Plant extraction has served as an initial and crucial procedure in ensuring the maintenance of the quality and safety of natural products for centuries. Extensive literature exploring the ideal extraction methods optimising the extractability of phytochemicals from plants, specifically *Garcinia*, was well defined. Yet, a lack of studies comparing the effects of direct and sequential extraction methods on these plants was indeed noticeable. This study evaluated the possible effects of various extraction procedures on the *Garcinia* species. Three *Garcinia* plants were selected, subjected to solvents under varying degrees, and extracted through direct and sequential techniques. The current results revealed that the direct extraction technique yields the highest in polyphenol compositions, including their biochemical activities, with ethanolic extract displaying the highest performance among all solvent polarities. In terms of antimicrobial activity, the ethanolic crude extract positively suppressed the inhibition of the growth of *Staphylococcus aureus* and *Bacillus subtilis* at specific concentrations. The

findings suggested utilising a direct extraction technique to improve the extraction quality of *Garcinia*, signifying its relevance and efficiency in obtaining the phytochemicals with enhanced antioxidants and antimicrobial properties.

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INTRODUCTION

Traditional and natural products have been embraced and relied upon across the globe since ancient times, serving as primary healthcare to approximately 85% of the worldwide population (World Health Organisation [WHO], 2013). In many Asian countries, medicinal products are applied immensely due to their full and deep-rooted cultural practices for generations, creating the foundation of Traditional, Complementary, and Alternative Medicine (TCAM). Malaysia, in particular, has shown consistent reliability towards the adoption of TCAM, with nearly 70% of the population applying it in treating ailments and diseases (Siti et al., 2009). The increment in global dependency on traditional remedies consistently induced advancements in natural product development nowadays, emphasising the need for ongoing enhancement of innovation in fields such as food technology, pharmaceuticals, nutraceuticals, and agriculture.

Medicinal and aromatic plants, specifically those with ethnopharmacological importance, have served as natural reservoirs of TCAM for millennia (Chaachouay & Zidane, 2024). These plants are acknowledged to exhibit a notable spectrum of natural bioactive compounds with various chemical structures and challenging dynamic properties (Wernisch & Pennathur, 2016). Regardless, their multifaceted matrices limit the expansion of research on natural products, hindering the availability of the compounds and reducing the extraction efficiency. Considering these aforementioned issues, optimising the extraction process as the initial foundation step remains a critical priority in any intended research or applications.

In the extraction procedure, achieving the optimum extraction efficiency requires maximising the extractable yield of plants. Extractable matter, which consists of a wide range of secondary metabolites, essentially relies on the choice of an appropriate solvent, which feasibly influences the specificity of the compounds extracted. Generally, extraction methods are designed to target either polar or non-polar compounds using solvents of varying polarities. In most extensive studies previously, secondary metabolites and their bioactivities are often in the limelight, neglecting the possibility of gaps within the extraction methods impacting the quality of the plant extracts. In this sense, it is imperative to explore and assess all potential methods to bridge this research gap effectively.

The *Garcinia* genus, affiliated with the Clusiaceae family, is in the spotlight for having an abundance of bioactive compounds such as bioflavonoids, polyphenols, and xanthenes possessing substantial therapeutic properties, including antioxidant and antimicrobial (Acuña et al., 2012; Santo et al., 2020). Attributable to their wide pharmacological potencies, numerous health and food products were developed, such as gels, creams, lotions, tablets, and syrups with *Garcinia* extracts as one of the ingredients, delivering a variety of health benefits to consumers (Krisanti et al., 2020; Paul & Zaman, 2022; Saptarini &

Hadisoebroto, 2020). Nevertheless, comparable to naturally produced products, therapeutic drugs that have been effectively developed pose several challenges (Harvey et al., 2015). It is worth mentioning that developing these natural products for direct consumption relies heavily on appropriate extraction procedures. With respect to *Garcinia*, wide-ranging research has been conducted to identify the best extraction technique yielding promising results over the years. Nevertheless, studies particularly comparing the effectiveness of sequential and direct extraction methods remain deficient.

The current study involved a comparative experiment analysing the variability of the polyphenolic compounds with few biological activities extracted from selected *Garcinia* plants, utilising two different solvent methods and analytical techniques (Figure 1). These plant species were chosen for their wide spectrum of pharmacological activities exhibited under various polarities. These materials were exposed to various methods with hexane, chloroform, and ethanol as extractants. Antioxidants with different scavenging assays and antimicrobials with various foodborne microorganisms were evaluated on the *Garcinia* extracts for their efficacies.

MATERIALS AND METHODS

Plant Preparations and Processing

For this study, three main *Garcinia* species were selected and collected at different areas within Universiti Putra Malaysia (Longitude: 101.7056 ° E, Latitude: 2.9995 ° N). *Garcinia mangostana* and *G. athroviridis* were harvested from Ladang Bersepadu, Ladang 10, whereas *G. hombroniana* was obtained from the Conservatory Garden of the Bioscience Institute. Leaves were collected from healthy plants at a comparable maturity stage to minimise variation in leaf age. Collection was performed within the same sampling period to minimise seasonal effects. The collected samples were first rinsed under running water to remove debris before being dried with tissue paper. Then, the samples were dried for 3 days consecutively at 30 °C in a conventional oven. Drying was conducted at a relatively low temperature of 30 °C to minimise thermal degradation of heat-sensitive compounds. Although some degradation cannot be fully excluded, the use of mild drying conditions was intended to reduce moisture while preserving phytochemical stability. After drying, the dried samples were ground into fine powders and stored properly at -20 °C for further research. The study was limited to three *Garcinia* species because these species are locally available, commonly used, and represent relevant members of the genus with reported phytochemical potential. Although the *Garcinia* genus is diverse, the selected species allowed a controlled comparative evaluation of extraction performance under the same experimental conditions.

Plant Extractions

A direct and sequential extraction process in this study was executed, in which fine-powdered *Garcinia* samples were precisely measured and macerated in various solvent polarities at a ratio of 1:10 (g/mL) (Figure 1). After 24 hours of macerating, the supernatant was obtained by filtering with Whatman filter No.1, demonstrating a direct extraction procedure. Proceeding to the sequential extraction required proper drying of the residue after the filtration by submerging it in the water bath at 40 °C, while the collected supernatants were concentrated at 40 °C with a rotary evaporator. The dried residue was correspondingly extracted through maceration with other solvents. All extracts were stored at -20 °C before they were used in the next analysis. In this study, maceration was selected because it is a simple, low-cost, and widely used extraction technique suitable for heat-sensitive plant bioactives such as phenolics and flavonoids. It also reflects a practical method that can be easily replicated without advanced equipment.

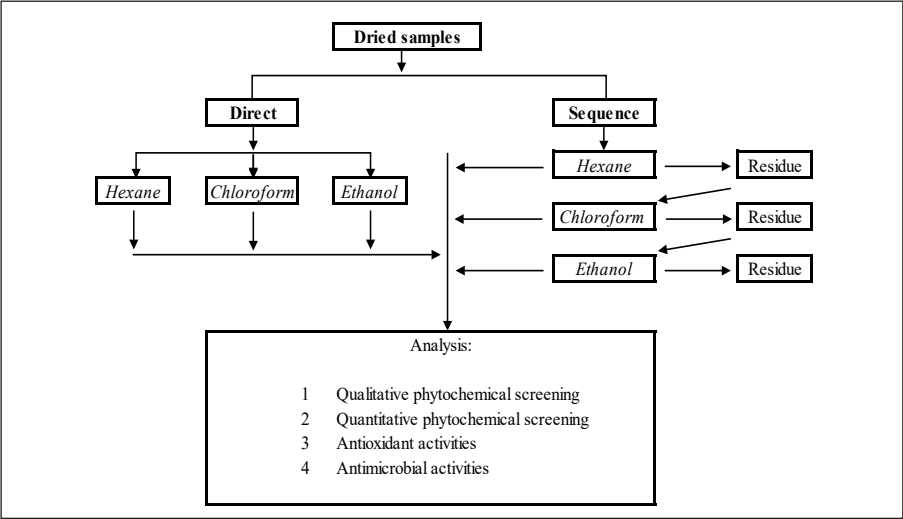


Figure 1. The flowchart of the extraction procedures subjected to the *Garcinia* samples

Extractable Matter and Phytochemical Screening Analysis

The extractable matter of *Garcinia* extracts was calculated as in Equation 1:

$$Extraction\ yield\ (\%) = \frac{Weight\ of\ crude\ extract}{Weight\ of\ powdered\ sample} \times 100 \qquad [1]$$

The phytochemical constituents, including phenolics, flavonoids, alkaloids, saponins, glycosides, tannins, and terpenoids in each plant crude extract were identified qualitatively according to the method described by Bisso et al. (2022).

Total Phenolic Content Determinations

The phenolic contents of *Garcinia* plant extracts were evaluated using the Folin-Ciocalteu method with slight adjustments (Samat et al., 2020). The samples with a volume of 200 μ L were mixed with 2 mL of Folin-Ciocalteu's phenol reagent and allowed to react for 10 minutes. After that, the mixture was added with 1 mL of sodium carbonate solution and left aside with an incubation period of 30 min in dark conditions. The mixtures were then measured under a microplate reader with 765 nm as an absorbance reading. The phenolic values were calculated and presented as mg gallic acid equivalent/ g dry extract with Gallic acid as standard.

Total Flavonoid Content Determinations

The flavonoid compositions for each *Garcinia* sample were measured following Samat et al. (2020) with minor modifications. First, diluted samples were added with 0.3 mL of sodium nitrite solution and left aside for 6 minutes. Then, 0.3 mL of aluminium chloride solution was added to the working samples and set aside for 6 minutes. Finally, 2 mL of sodium hydroxide (1M) was added and left aside for another 20 minutes for the reaction. The samples were then measured under a microplate reader with an absorbance of 510 nm. The flavonoid values were quantified and presented as quercetin equivalent/ g dry extract.

DPPH Scavenging Activity

The radical scavenging activity exhibited by *Garcinia* samples was evaluated using the 2,2-diphenyl-picrylhydrazyl (DPPH) radical scavenging assay (Samat et al., 2020). About 1 mL of each sample was mixed with 1 mL of working DPPH solution prepared freshly with methanol (1M). The colour changes after 30 min dark incubations indicate the presence of antioxidants, and their values were measured at 517 nm using the following Equation 2:

$$DPPH \text{ scavenging inhibition } (\%) = \frac{(Abs_{1517} - Abs_{0517})}{Abs_{0517}} \times 100 \quad [2]$$

where, Abs0 and Abs1 were the absorbances for the control and sample, respectively.

ABTS Scavenging Activity

The scavenging activity through ABTS was done by preparing 7 mM ABTS in distilled water and 2.45 mM potassium persulfate in a ratio of 1 to 1, forming the ABTS working solution before being placed under a dark ambient room for 12-16 hours. Prior to usage, the mixture of ABTS was initially diluted with methanol until obtaining an absorbance of 0.700 at 734 nm. Then, 1 mL of diluted ABTS was mixed with 3 mL of each of the samples and incubated for 30 minutes. After the incubation, the reading was measured

using a microplate spectrophotometer. Percent inhibition of absorbance at 734 nm was calculated using Equation 3:

$$ABTS \text{ scavenging inhibition (\%)} = \frac{(AB - AA)}{AB} \times 100 \quad [3]$$

where, AB is the absorbance of ABTS radical + methanol; AA is the absorbance of ABTS radical + sample extract/standard.

Antimicrobial Analysis

The antimicrobial activity of the *Garcinia* samples was evaluated on the microbial test strains, including *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, which were obtained from the Institute of Bioscience situated in the UPM. For the culturing media, nutrient agar and Mueller-Hinton Agar (MHA) were utilised to analyse the respective minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC). The assessment of the microbial assay of the samples was executed by using the agar dilution method. For the execution of MIC and MBC, various concentrations were tested until the concentration that completely suppressed the bacterial growth after 48 hours of incubation at 37 °C.

Statistical Analysis and Interpretation

The means of data collected in this study were analysed with one-way ANOVA (SAS 13.1) and the mean separation of the data was done using Tukey's Honestly Significant Difference (HSD) Test.

RESULTS

Extraction Yields and Qualitative Phytochemical Analysis

As presented in Figure 2, the extractable matter of *Garcinia* samples varied significantly following *Garcinia* species and solvent polarities. For plant species, *G. mangostana* produced the highest crude extract in comparison to *G. hombroniana* and *G. atroviridis*. Among the extraction methods, it was observed that the direct extraction technique effectively aids in yielding the extractable matter of *Garcinia*, approximately higher than the sequential method, about 30%, which varies across solvent polarities and *Garcinia* species categories. As we compared the effectiveness of the solvent polarities, it was demonstrated that crude extracts were influenced substantially by the type of solvents, with crude extract through ethanol as the most extractive sample, with a considerable average value of 20% in the direct extraction technique and 14% in the sequential extraction method.

From the perspective of phytochemicals in a qualitative sense, insignificant variations in the appearance of phytochemicals can be detected between *Garcinia* species in either direct or sequential extraction techniques. Most polar compounds were found in ethanol and chloroform, whereas non-polar compounds were detected heavily in hexane. With the exception of saponin, which was solely identified through the sequential method, the detection trend of phytochemical compounds was largely consistent across both extraction techniques (Table 1).

Quantitative Phytochemical Analysis

Figures 3 and 4 illustrate significant variations in phenolic and flavonoid contents across all species and solvent polarities when using direct and sequential extraction methods. According to the table, phenolic (Figure 3) and flavonoid (Figure 4) concentrations decreased by 15% to 35% during sequential extraction, regardless of solvent polarity. Similar to the extractive values, polyphenolic compounds were most abundant in *G. mangostana* when extracted with the most polar solvents, followed by *G. hombroniana* and *G. atroviridis*. In contrast, the lowest polyphenolic content was consistently observed in hexane across all species and extraction methods. In all species, flavonoid concentrations were approximately 30% to 60% lower than phenolic concentrations, irrespective of the solvent polarity or extraction method employed.

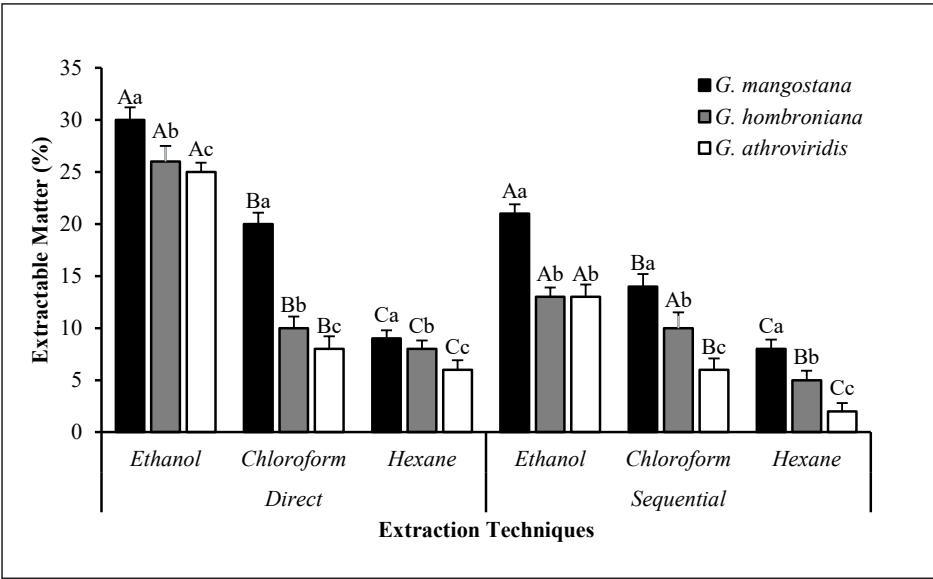


Figure 2. The extractable yield of *Garcinia* at different solvent polarities and extraction techniques
Note. Each bar chart represents the means ± standard error. Means followed by different letters (A, B, C) and (a, b, c) are significantly different at P<0.05 within the type of solvents and plant species, respectively

Table 1
Qualitative phytochemical assessment of *Garcinia* species subjected to direct and sequential extraction methods

Extraction Techniques	Extractants	<i>Garcinia</i> Species	Phytochemicals						
			Flavonoids	Phenolics	Alkaloids	Saponins	Tannins	Terpenoids	Glycosides
Direct	Ethanol	<i>Garcinia hombroniana</i>	+++	+++	+++	+++	+++	+	+
	Chloroform		++	++	+	++	++	+	++
	Hexane		-	-	-	-	-	++	++
Sequential	Ethanol		+++	+++	+++	+++	+++	-	+
	Chloroform		++	++	+	+	+	++	+
	Hexane		-	-	-	-	-	++	+
Direct	Ethanol	<i>Garcinia mangostana</i>	+++	+++	+++	+++	+++	+	+
	Chloroform		+++	++	++	++	++	+	++
	Hexane		-	-	-	-	-	+++	++
Sequential	Ethanol		+++	+++	+++	+++	+++	+	+
	Chloroform		++	++	++	++	++	++	++
	Hexane		-	-	-	-	-	++	++
Direct	Ethanol	<i>Garcinia athroviridis</i>	+++	+++	++	++	++	++	+
	Chloroform		+	+	+	+	+	++	+
	Hexane		-	-	-	-	-	++	+
Sequential	Ethanol		++	++	++	++	++	+	+
	Chloroform		+	+	+	+	+	++	+
	Hexane		-	-	-	-	-	++	+

Notes. (+) = Present in small concentration, (++) = Present in moderately high concentration, (+++) = Present in high concentration, (-) Absent

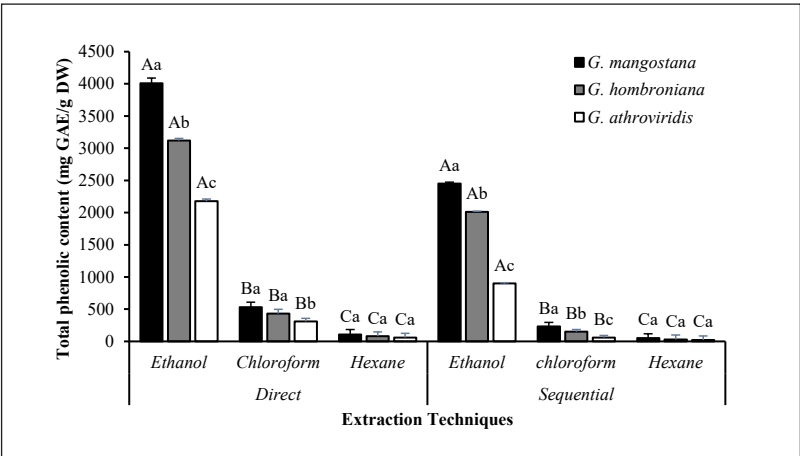


Figure 3. The phenolic contents of *Garcinia* at different solvent polarities and extraction techniques
Note. Each bar chart represents the means $\pm$ standard error. Means followed by different letters (A, B, C) and (a, b, c) are significantly different at $P < 0.05$ within the type of solvents and plant species, respectively

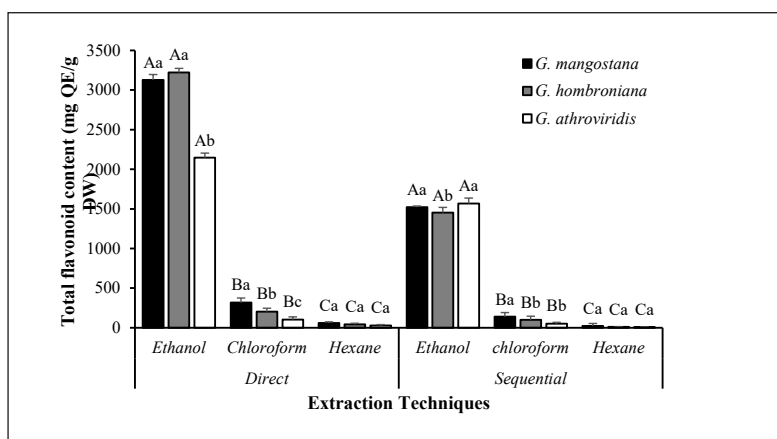


Figure 4. The flavonoid contents of *Garcinia* at different solvent polarities and extraction techniques
 Note. Each bar chart represents the means $\pm$ standard error. Means followed by different letters (A, B, C) and (a, b, c) are significantly different at $P < 0.05$ within the type of solvents and plant species, respectively

Radical Scavenging Activity

The antioxidant properties of plants can be evaluated through two primary mechanisms known as single electron transfer (SET) and hydrogen atom transfer (HAT). In this study, the antioxidant activity of the plants was assessed using DPPH and ABTS assays, as shown in Figures 5 and 6. These assays were used to investigate how the extraction methods and solvent polarities influenced the antioxidant capacity of the extracts.

The scavenging activity of all extracts differed depending on the extraction methods and solvent polarities, as depicted in Figures 5 and 6. Overall, the difference in antioxidant percentage between the DPPH and ABTS assays was minimal. A pattern similar to the polyphenolic compositions was observed, where extracts obtained directly from the plants exhibited higher antioxidant activity compared to those obtained using the sequential extraction method. Among the samples, *G. mangostana* showed the strongest scavenging activity when extracted with polar solvents, while *G. atroviridis* consistently displayed the lowest antioxidant activity across all extraction methods. Additionally, ethanolic extracts demonstrated significantly higher inhibition values, approximately 50% greater than those of chloroform extracts, irrespective of the species or extraction technique.

Antimicrobial Activity Test

According to Table 2, ethanolic extracts from all species were tested at a concentration of 100 mg/mL to evaluate their antimicrobial activity. The results revealed that the ethanolic extracts exhibited notable activity against *Staphylococcus aureus* and *Bacillus subtilis* compared to other microorganisms in the study, as shown in Figure 7. In contrast, extracts obtained using chloroform and hexane demonstrated no activity against any of the tested microorganisms at the same concentration.

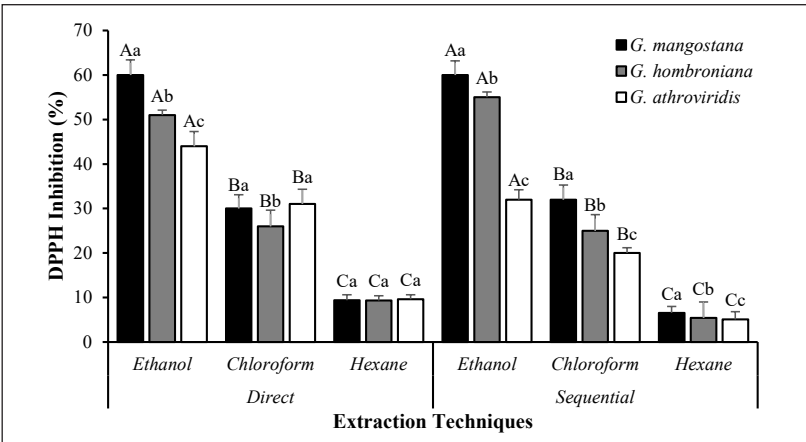


Figure 5. The DPPH scavenging activity of *Garcinia* at different solvent polarities and extraction techniques
Note. Each bar chart represents the means $\pm$ standard error. Means followed by different letters (A, B, C) and (a, b, c) are significantly different at $P < 0.05$ within the type of solvents and plant species, respectively

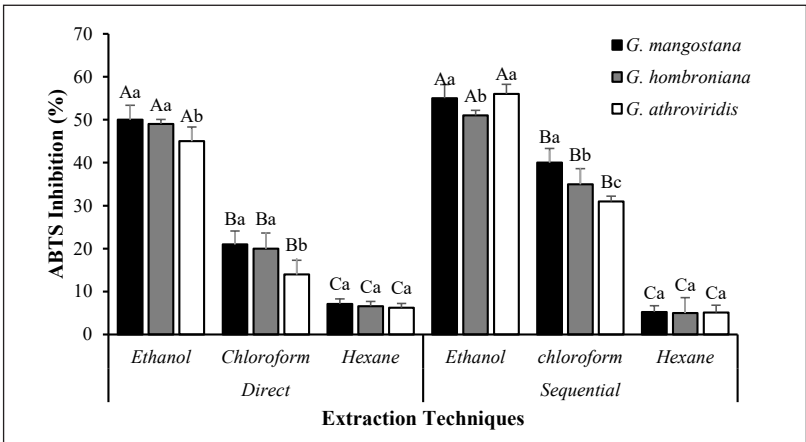


Figure 6. The ABTS scavenging activity of *Garcinia* at different solvent polarities and extraction techniques
Note. Each bar chart represents the means $\pm$ standard error. Means followed by different letters (A, B, C) and (a, b, c) are significantly different at $P < 0.05$ within the type of solvents and plant species, respectively

Table 2
The antimicrobial properties of *G. mangostana* extracted through the direct method

Extractants	<i>Pseudomonas aeruginosa</i> (ATCC 15422)	<i>Escherichia coli</i> (ATCC 25922)	<i>Bacillus subtilis</i> (B29)	<i>Staphylococcus aureus</i> (ATCC 43300)
Ethanol	0	0	11.57 $\pm$ 2.14	11.37 $\pm$ 2.45
Chloroform	0	0	0	0
Hexane	0	0	0	0

Note. Data presented as mean with triplicate $\pm$ standard error. 0 value indicates no inhibition expressed upon solvent application

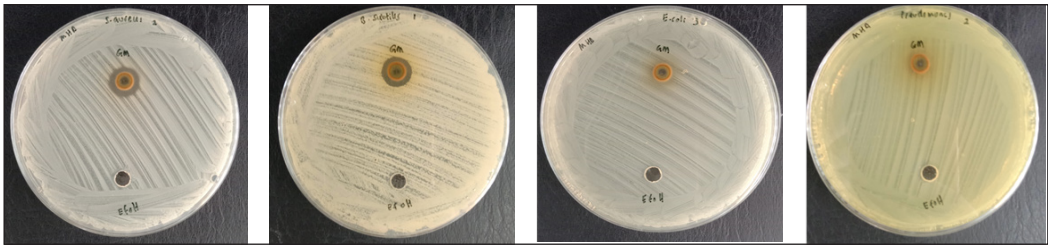


Figure 7. Well diffusion test of microbial suppression of *G. mangostana* extracts was exhibited only by *S. aureus* and *B. subtilis*

Table 3 presents the MIC values of the ethanolic extracts. Hexane and chloroform extracts showed no activity across all tested concentrations (data not shown). The ethanolic extract exhibited inhibitory effects against *S. aureus* at concentrations ranging from 25 to 100 mg/mL, while for *B. subtilis*, inhibition was observed between 50 and 100 mg/mL. The MBC results were consistent with the MIC, as no bacterial growth was detected at the lowest MIC values. Specifically, the ethanolic extract demonstrated the strongest suppression of *S. aureus* at 25 mg/mL, whereas the MBC for *B. subtilis* was determined to be 50 mg/mL.

Table 3
Minimum inhibition concentration (MIC) of the ethanolic extract of *G. mangostana*

Extractant	Concentration (mg/mL)	Inhibition Zone (mm)	
		<i>S. aureus</i>	<i>B. subtilis</i>
Ethanol	6.25	0	0
	12.5	0	0
	25	9 ± 1.6	0
	50	10 ± 1.8	11 ± 0.6
	100	12 ± 1.7	11 ± 1.2

Note. Data are means with triplicates ± standard error. 0 indicates no inhibition occurred upon solvent application

DISCUSSION

Since ancient times, the perspective of plants being the reservoir of valuable and natural bioactive phytochemicals has been fully embraced and incorporated into modern medicines for the treatment of various human diseases and ailments. In addressing chronic health conditions, herbal medicines offer substantial benefits, including potency effect, cost-effectiveness, minimal side effects, and ease of access. Regardless of their significance, these plants existed in multifaceted and complex matrices, which complicate the extraction process of specific bioactive compounds. Considering these issues, numerous approaches have been proposed, and it is plausible that emphasising the optimisation of the extraction

process will feasibly be the answer for the enhancement of the quality of the medicinal plant extracts.

Many exemplary works of literature successfully defined the substantial effect of extractant selection and extraction technique on the retrieval of polyphenols and their biochemical properties (Dirar et al., 2019; Ngo et al., 2017). In the recovery of the polyphenols, the impact of the type of extractants is more noticeable than the extraction technique itself in the present findings, regardless of the *Garcinia* species. These results were accurate since solvent polarity influences bioactive compound extraction efficiency (Wado et al., 2022). Arawande et al. (2018) also added that these solvents' ability to extract selected compounds can be evaluated based on the extractable crude matter collected from the extraction. Crude extract of chloroform, for instance, belongs to a non-polar solvent but exhibits an uneven charge distribution in its molecular structure, allowing it to attract polar compounds comparable to water (Martinsen & Heiskanen, 2023). This occurrence explained the lower polyphenolic compositions in the sequential technique when ethanol was used as the solvent, due to some compounds being feasibly extracted by chloroform beforehand. In contrast to chloroform, the presence of a polar hydroxyl group in the ethanol structure contributes to a high net dipole moment, increasing hydrogen bonding ability with other molecules. This property enables ethanol to attract more polar compounds, accounting for the significant increase in polyphenolic compound extraction observed in Figures 3 and 4.

Phytochemical analysis of *G. mangostana* leaf extract, along with other *Garcinia* species (data not shown), revealed the presence of various phytochemical compounds in both chloroform and ethanolic extracts. While both extracts demonstrated antioxidant properties, antimicrobial activity was observed only in the ethanolic extract, with hexane showing no activity against any tested microorganisms. These findings differ from those of Bhat and Al-Daihan (2013) and Janardhanan et al. (2020), who reported significant antimicrobial activity in the chloroform extract of *G. mangostana*. The inconsistency in findings could be attributable to the differences in extraction technique, given the low polarity of chloroform compared to ethanol. Accordingly, ethanol feasibly dissolved more active antimicrobial phytochemicals, reducing the effectiveness of the chloroform extract. Moreover, it is worth mentioning that the variability in polyphenol patterns in this study is probably due to the usage of crude extract form, which exhibits limitations. The potential for synergistic or antagonistic effects expressed by multiple bioactive compounds highlights the significance of evaluating the interactions between these compounds.

CONCLUSION

The direct extraction technique was revealed to improve the productivity of phytochemicals and extractable matter specifically for *G. mangostana*. Incomparable to the sequential technique, the direct extraction technique effectively enhanced the radical scavenging

activity of *Garcinia* plants, evidenced by the greater findings of DPPH and ABTS activity. Future studies emphasise identifying particular bioactive compounds accountable for the expression of antioxidants and antimicrobial activities in direct and sequential techniques, potentially offering a more comprehensive understanding of the overall variability of the results obtained.

ACKNOWLEDGEMENT

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REFERENCES

- Acuña, U. M., Dastmalchi, K., Basile, M. J., & Kennelly, E. J. (2012). Quantitative high-performance liquid chromatography photodiode array (HPLC-PDA) analysis of benzophenones and bioflavonoids in eight *Garcinia* species. *Journal of Food Composition and Analysis*, 25(2), 215–220. <https://doi.org/10.1016/j.jfca.2011.10.006>
- Arawande, J. O., Alademeyin, J. O., Akinnusotu, A., & Alademeyin, J. O. (2018). Extractive value and phytochemical screening of ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) using different solvents. *Traditional Natural Medicine*, 8(1), 13–22.
- Bhat, R. S., & Al-Daihan, S. (2013). Antimicrobial activity of *Garcinia mangostana* using different solvent extracts. *International Journal of Biosciences*, 3(10), 267–272. <https://doi.org/10.12692/ijb/3.10.267-272>
- Bisso, B. N., Kayoka-Kabongo, P. N., Tchuenteu, R. T., & Dzoyen, J. P. (2022). Phytochemical analysis and antifungal potentiating activity of extracts from loquat (*Eriobotrya japonica*) against *Cryptococcus neoformans* clinical isolates. *Advances in Pharmacological and Pharmaceutical Sciences*, 2022(1), Article 6626834. <https://doi.org/10.1155/2022/6626834>
- Chaachouay, N., & Zidane, L. (2024). Plant-derived natural products: A source for drug discovery and development. *Drugs and Drug Candidates*, 3(1), 184–207. <https://doi.org/10.3390/ddc3010011>
- Dirar, A. I., Alsaadi, D. H. M., Wada, M., Mohamed, M. A., Watanabe, T., & Devkota, H. P. (2019). Effects of extraction solvents on total phenolic and flavonoid contents and biological activities of extracts from Sudanese medicinal plants. *South African Journal of Botany*, 120, 261–267. <https://doi.org/10.1016/j.sajb.2018.07.003>
- Harvey, A. L., Edrada-Ebel, R., & Quinn, R. J. (2015). The re-emergence of natural products for drug discovery in the genomics era. *Nature Reviews Drug Discovery*, 14, 111–129. <https://doi.org/10.1038/nrd4510>
- Janardhanan, S., Mahendra, J., Mahendra, L., & Devarajan, N. (2020). Cytotoxic effects of mangosteen pericarp extracts on oral cancer and cervical cancer cells. *Asian Pacific Journal of Cancer Prevention*, 21(9), 2577–2583. <https://doi.org/10.31557/APJCP.2020.21.9.2577>
- Krisanti, E. A., Farizal, A. N., & Mulia, K. (2020). *Garcinia mangostana* L. fruit rind extract in ethyl acetate, n-butanol, and water fractions: Phytochemical analysis, antioxidant assay, and cytotoxicity assay. *IOP Conference Series: Materials Science and Engineering*, 1053, Article 012040. <https://doi.org/10.1088/1757-899X/1053/1/012040>

- Martinsen, O. G., & Heiskanen, A. (2023). *Bioimpedance and bioelectricity basics* (4th ed.). Elsevier. <https://doi.org/10.1016/B978-0-12-819107-1.00004-2>
- Ngo, T. V., Scarlett, C. J., Bowyer, M. C., Ngo, P. D., & Vuong, Q. V. (2017). Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *Journal of Food Quality*, 2017, Article 9305047. <https://doi.org/10.1155/2017/9305047>
- Paul, A., & Zaman, M. K. (2022). A comprehensive review on ethnobotany, nutritional values, phytochemistry and pharmacological attributes of ten potent *Garcinia* species of South-east Asia. *South African Journal of Botany*, 148, 39–59. <https://doi.org/10.1016/j.sajb.2022.03.032>
- Samat, N. M. A. A., Ahmad, S., Awang, Y., Bakar, R. A. H., & Hakiman, M. (2020). Alterations in herbage yield, antioxidant activities, phytochemical contents, and bioactive compounds of Sabah snake grass (*Clinacanthus nutans* L.) with regards to harvesting age and harvesting frequency. *Molecules*, 25(12), Article 2833. <https://doi.org/10.3390/molecules25122833>
- Santo, B. L. S. D., Santana, L. F., Junior, W. H. K., Araujo, F. D. O. D., Bogo, D., Freitas, K. D. C., Guimaraes, R. D. A., Hiane, P. A., Pott, A., Filiu, W. F. D. O., Asato, M. A., Figueiredo, P. D. O., & Bastos, P. R. H. D. (2020). Medicinal potential of *Garcinia* species and their compounds. *Molecules*, 25(19), Article 4513. <https://doi.org/10.3390/molecules25194513>
- Saptarini, N. M., & Hadisoebroto, G. (2020). Formulation and evaluation of lotion and cream of nanosized chitosan-mangosteen (*Garcinia mangostana* L.) pericarp extract. *Rasayan Journal of Chemistry*, 13(2), 789–795. <https://doi.org/10.31788/RJC.2020.1325533>
- Siti, Z. M., Tahir, A., Farah, A. I., Ami Fazlin, S. M., Sondi, S., Azman, A. H., Maimunah, A. H., Haniza, M. A., Siti Haslinda, M. D., Zulkarnain, A. K., Zakiah, I., & Wan Zaleha, W. C. (2009). Use of traditional and complementary medicine in Malaysia: A baseline study. *Complementary Therapies in Medicine*, 17(5–6), 292–299. <https://doi.org/10.1016/j.ctim.2009.04.002>
- Wado, T. E., Suleman, S., & Mohammed, T. (2022). Antimicrobial evaluation of sequentially extracted leaf of *Vernonia auriculifera* Hiern. *BMC Complementary Medicine and Therapies*, 22, Article 219. <https://doi.org/10.1186/s12906-022-03690-2>
- Wernisch, S., & Pennathur, S. (2016). Evaluation of coverage, retention patterns, and selectivity of seven liquid chromatographic methods for metabolomics. *Analytical and Bioanalytical Chemistry*, 408(22), 6079–6091. <https://doi.org/10.1007/s00216-016-9716-4>
- World Health Organisation (WHO). (2013). *World Health Organisation traditional medicine strategy: 2014-2023*.

Anti-inflammatory, Antioxidant and Antimicrobial Activities of Extracts from Ecolite® Waist Tonic

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ABSTRACT

Waist tonics are commonly used as a traditional remedy for pain relief, energy enhancement, and overall health improvement. Ecolite® Waist Tonic (EWT) extract contains the traditional herbal ingredients used in waist tonic. With reported over 500,000 cases of low back pain worldwide in 2020, with a projection of 843 million by 2050, the demand for effective medication is urgent. The use of COX-2 inhibitors is essential in managing inflammation, particularly in conditions like back pain. Therefore, this study sought to evaluate the *in vitro* COX-2 inhibitory activity together

with antioxidant and antibacterial activities of EWT extract to understand the scientific basis for its traditional use in managing pain and inflammation. COX-2 inhibitory activity exhibited EWT extract was evaluated using an enzyme-based assay. Additionally, FRAP, DPPH and ABTS assays were used to assess the antioxidant activity of EWT extract, whereas antimicrobial activity was tested against two pathogenic bacteria and two fungi. Although the moderate antioxidant activity and weak antimicrobial properties exhibited by EWT extract, a strong COX-2 inhibition of 72% was found in EWT extract, which is comparable with

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77% inhibition of celecoxib as a positive control. These results support its potential use in managing and controlling inflammation.

Keywords: Antioxidant activity, antimicrobial activity, COX-2 inhibition, Ecolite® waist tonic, traditional Chinese medicine

INTRODUCTION

Low back pain (LBP) is ranked in the top ten most frequently attended illnesses by the primary healthcare professionals (Kuritzky & Samraj, 2012). About 70% of the world population complained of suffering acute or chronic back pain throughout their lifetime (Strine & Hootman), which resulted in 619 million cases of LBP reported worldwide in 2020. In a projection towards 2050, there will be an estimated 843 million people worldwide who will suffer LBP (Ferreira et al., 2023). In Malaysia, about 12% of the total population, or 3.8 million Malaysians, are reported to acquire LBP (Sivasampu et al., 2014). There is a significantly higher rate of LBP cases found among the high-risk groups. The prevalence of LBP has been reported to affect Malaysian, ranging from 12.4% to 84.6% across different groups of residents (Daud et al., 2023). It is expected to rise over the years, as a result of the changes in lifestyle after the COVID-19 pandemic, such as work-from-home policies and post-pandemic social practice alterations (Muniandy et al., 2022). The elevating trend of LBP cases resulted in societal and economic burden, particularly in the low- and middle-income countries, as it causes increasing direct and indirect healthcare costs. In addition, the impact also propagated to the incremental early retirement personnel due to the frequent absenteeism at work and the depletion in work productivity (Hartvigsen et al., 2018). It is therefore a dire need to seek solutions in managing and preventing LBP.

Oxidative stress, microbial infections, and inflammation can be classified as the main causes of many health complications. Antioxidants are known to neutralise free radicals that are associated with chronic diseases (Lobo et al., 2010), while antimicrobial agents can help to combat infections. Cyclooxygenase (COX)-2 inhibitors are crucial in managing inflammation, making them useful in treating conditions such as arthritis and back pain (Fine, 2002). The treatment of LBP with COX-2 inhibitors is well-established, but can be a complicated topic in pain management. COX-2 inhibitors (the coxibs series) are a subclass of nonsteroidal anti-inflammatory drugs (NSAIDs) that were developed to offer the pain-relieving benefits of traditional NSAIDs (van der Gaag et al., 2020). Although the use of coxibs series has a reduced risk of gastrointestinal (GI) side effects, there are reports of increased risk of cardiovascular events, including heart attack and stroke, which led to the withdrawal of rofecoxib (Vioxx) and valdecoxib (Bextra) from the market (Bennett et al., 2005).

Ecolite® Waist Tonic (EWT) is a natural herbal formulation crafted with a combination of traditional Chinese herbs including *Eucommia ulmoides* (du zhong), *Achyranthes bidentata* (Niu xi), *Panax ginseng* (ren shen), *Cordyceps sinensis* (dong chong xia cao), *Ganoderma lucidum* (ling zhi), *Psoralea corylifolia* (buguzhi) and *Lycium Barbarum* (Gouqizi), in combination with other 10 ingredients, namely *Ligusticum chuanxiong*, *Polygonatum sibiricum*, *Morinda officinalis*, *Rehmannia glutinosa*, *Salvia miltiorrhiza*, *Citrus reticulata*, *Schisandra chinensis*, *Sophora flavescens*, *Citrus aurantium*, and *Glycyrrhiza uralensis*. A standardised tongkat ali extract (*Eurycoma longifolia*) is also added to this remedy to elevate the overall functional properties as a traditional local herb. (NPRA, 2025). EWT is made to alleviate lower back pain, boost energy and vitality, combat fatigue, enhance blood circulation and support overall health.

In the effort of seeking a potential supportive LBP management alternative, in this study, we focused on the evaluation of COX-2 inhibitory activities in complement with antioxidant and antimicrobial activities of EWT extract via *in vitro* assays, offering insights into its potential applications in modern healthcare.

MATERIALS AND METHODS

Materials

EWT was provided by Ecolite Biotech Manufacturing Inc. as blended plant extracts in aqueous form (4% w/v). The extracts were freeze-dried prior to further analysis. Chemicals used for this work include Elabscience COX-2 Inhibitor Screening kits (E-BC-D002) and four positive controls or reference drugs (celecoxib, quercetin, nystatin and streptomycin sulfate), ferrous sulfate (FeSO_4), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), sodium acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), dimethyl sulfoxide (DMSO), Pathogenic bacterial and fungal strains used for antimicrobial tests were including *Bacillus subtilis*, *Streptococcus pyogenes*, *Candida albicans* and *Aspergillus niger*.

Experimental Setup

COX-2 Inhibition Assay

This assay was conducted according to the test protocols provided by the Elabscience COX-2 inhibition assay kit (E-BC-D002). The assay also utilised a water bath, an analytical balance, VANTASTAR multi-mode microplate reader (BMG LABTECH), micropipette and microcentrifuge tubes. EWT solution (400 mg/mL) was prepared by dissolving 400 mg Ecolite extract in 1 mL dimethyl sulfoxide (DMSO). Working concentrations were prepared by subsequent dilution in DMSO to yield final concentrations of 200 mg/mL and 100 mg/mL, respectively. The accelerator, chromogen and substrate solution were mixed thoroughly

and frozen before use of the kit. The reagents were protected from light for the whole experiment. Celecoxib (purity >98%, 38 µg/mL) was used to replace the tested sample solution as a positive control. The blank containing only the assay buffer was employed as the background reference for the measurement of fluorescence. The negative control group was the group containing recombinant COX-2 enzyme without any inhibitor and was set to be the maximum or 100% initial enzyme activity. The experiment was performed on a black 96-well microplate. Initially, 140 µL of assay buffer was pipetted into each well prepared for assay. 10 µL of the test sample solution and 10 µL of the positive control solution of celecoxib were added to the respective wells. 10 µL assay buffer was added to the negative control wells in place of the inhibitor solution. Subsequently, 20 µL of recombinant COX-2 enzyme solution was added to all wells except blank wells. The reaction mixture was then incubated at room temperature for 5 min to enhance the interaction of the inhibitor with the enzyme. After incubation, 10 µL of the chromogenic agent and 10 µL of the accelerator were added to each well. Later, 10 µL of substrate solution was added to each well to activate the enzymatic reaction. The plate was gently shaken and incubated in the dark at 37 °C for 30 minutes. The fluorescence intensity was then immediately measured with a fluorescence microplate reader (excitation and emission wavelengths were 535 nm and 587 nm, respectively). The inhibition percentages were calculated by comparing the absorbance values of the tonic extracts to those of untreated control enzymes.

Antioxidant Activity

The antioxidant activity of the waist tonic extracts was evaluated using three complementary assays, i.e., ferric reducing antioxidant power (FRAP) assay, DPPH radical scavenging assay and ABTS assay. Quercetin (≥95% purity via HPLC) was used as a positive control in all three assays.

FRAP Assay

The ferric reducing antioxidant power (FRAP) assay was performed following the previous method with modifications (Benzie & Strain, 1996). 10 µL of methanolic extract at two various concentrations (1 and 10 mg/mL; three replicates per sample and concentration) was mixed with 300 µL of freshly prepared FRAP reagent. The FRAP reagent was prepared from 10 parts of 300 mM sodium acetate buffer (pH 3.6), 1 part of 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), and 1 part of 20 mM FeCl₃ hexahydrate. The mixture was incubated at 37 °C for about 4 minutes. A microplate reader (Versamax™) is used to measure the sample absorbance at 470 nm. A blank control was prepared by replacing the methanolic extract with methanol. The results were expressed as milligram equivalents of FeSO₄ per milligram of dry extract weight. The assay was carried out in triplicate.

DPPH Radical Scavenging Assay

The DPPH assay was carried out using a published method with slight modifications (Güllüce et al., 2003). Ten milligrams (mg) of EWT extract was dissolved in 1 mL of methanol. This stock solution was then further diluted with methanol to ten different concentrations (0.0195, 0.0391, 0.0781, 0.1563, 0.3125, 0.6250, 1.2500, 2.5000, 5.0000 and 10.0000 mg/mL). The samples were then centrifuged at 10000 rpm for three minutes at 4 °C (Eppendorf 5415R centrifuge, Hamburg, Germany). 100 µl of each diluted sample was mixed with 100 µl of a 0.1 mg/mL DPPH solution in methanol in the 96-well plates and incubated at room temperature for 30 minutes. Their absorbance was subsequently obtained at 517 nm against a blank using a VersaMax™ microplate reader. The percentage of DPPH radical inhibition (I%) was calculated using Equation 1:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100 \quad [1]$$

Where A_{blank} is the absorbance of the control reaction that contains all reagents except the test compound, and A_{sample} is the absorbance of the test compound. The 50% inhibition (IC_{50}) of the extract was determined by plotting the percentage inhibition against the extract concentrations and was calculated from the resulting curve. The assay was carried out in triplicate.

ABTS Assay

The antioxidant activity of the extracts against $ABTS^{+\bullet}$ radicals was carried out using the method that has been published previously (Stratil et al., 2006). The $ABTS^{+\bullet}$ radical cation was initiated by mixing 7 mM $ABTS^{+\bullet}$ with 4.95 mM potassium persulfate in a 1:1 (v/v) ratio. This mixture was then kept in the dark for 12 hours at room temperature. The resulting solution was then diluted with methanol until its absorbance hit between 0.6 and 0.7 at 734 nm wavelength. Ten milligrams (mg) of the samples were dissolved in 1 mL of methanol. This stock solution was then further diluted with methanol to ten different concentrations (0.0195, 0.0391, 0.0781, 0.1563, 0.3125, 0.6250, 1.2500, 2.5000, 5.0000 and 10.0000 mg/mL). 10 µl of these diluted samples was transferred into a 96-well plate and added to 300 µl of $ABTS^{+\bullet}$ solution. The plate was incubated for 10 minutes at room temperature, and the absorbance was read at 743 nm wavelength using a VersaMax™ microplate reader. Methanol was used as a blank. A blank control was prepared using $ABTS^{+\bullet}$ solution, and results were expressed as percent inhibition. Quercetin was used as a positive control by experimenting with its series of concentrations ranging from 0.0020 mg/mL to 1 mg/mL. The assay was carried out in triplicate.

Antimicrobial Activity

Disk Diffusion Assay

The freeze-dried EWT extracts were dissolved in DMSO to a final concentration of 400 mg/mL and filtered through 0.45 µm Millipore filters. Antimicrobial activity was assessed using the disk diffusion method (Güllüce et al., 2003). A suspension of 10^8 CFU/mL of bacteria or 10^6 CFU/mL of fungi was spread onto nutrient agar (NA) or potato dextrose agar (PDA) media, respectively. Disks (6 mm in diameter) were impregnated with 400 mg/mL of the EWT extracts prepared earlier and placed on the inoculated agar. Negative controls were prepared using the solvent blank, i.e. DMSO. Streptomycin sulfate (1 mg/mL) was used as a positive control for bacterial strains, whereas nystatin (100 IU/disk) was used as a positive control for fungal strains. In the case of the antibacterial study, the inoculated plates were incubated for 24 hours at 37 °C, whereas in the case of the antifungal study, the inoculated plates were incubated for 48 hours at 30 °C. These antimicrobial strengths were evaluated by measuring the diameter of their resulting inhibition zones around the disks and were compared with the negative and positive controls. Each assay was carried out in triplicate.

Statistical Analysis

Data was analysed using the Statistical Package for Social Sciences (SPSS) (version 30.0) software. Statistical analysis was conducted using one-way ANOVA (analysis of variance) to compare the means of different groups. All experiments were carried out in triplicate, and the results are expressed as the mean $\pm$ standard deviation (SD). In the case of the COX-2 inhibition assay, a one-way ANOVA was performed to compare the mean of each EWT concentration column with the mean of the control column, followed by Dunnett's multiple comparison tests to determine their statistical significance, where they were denoted as $p < 0.05$, $p < 0.001$, and $**p < 0.0001$.

Normality Testing and QQ Plot Analysis

All data of COX-2 inhibitory activity were tested for normality with the GraphPad Prism 9.3.1 software package before the statistical analysis. Percentage inhibition data were calculated from triplicate experiments ($n = 3$) for positive control and EWT samples (100, 200 and 400 mg/mL) and were entered into GraphPad Prism. The “Column statistics” and “Normality and Lognormality Tests” in GraphPad Prism were used to examine the data for normality. The software produced a Normal Quantile-Quantile (QQ) plot, which compared the observed data distribution against the expected theoretical normal distribution. The QQ plot was used to plot the actual observed values against the predicted normal values, and normalcy was assessed by how close the data points were to the reference diagonal line.

RESULTS AND DISCUSSION

COX-2 Inhibitory Activity

As demonstrated in Table 1 and Figure 1, the EWT extract was found to exhibit COX-2 inhibitory activity in a concentration-dependent manner. In addition, the standard QQ plot shown in Figure 2 indicates that most data points of the EWT extract groups and positive control groups converged near the reference diagonal line. These analyses imply that data were generally distributed in a normal manner and were suited for parametric statistical analysis with one-way ANOVA followed by Dunnett’s multiple comparison tests.

Table 1
Percentage of COX-2 inhibition by Ecolite® Waist Tonic (EWT)

Sample	Percent Inhibition (%)
Celecoxib (Positive Control)	76.73 ± 0.86
400 mg/mL EWT	72.42 ± 2.43*
200 mg/mL EWT	70.53 ± 1.10**
100 mg/mL EWT	65.91 ± 1.76***

Note. Values are expressed as Mean ± SD (n=3). *p<0.05, **p<0.001, and ***p<0.0001, indicating they are statistically significant. One-way ANOVA (analysis of variance) was performed to compare the mean of each EWT concentration with the mean of the positive control column, followed by Dunnett’s multiple comparison tests

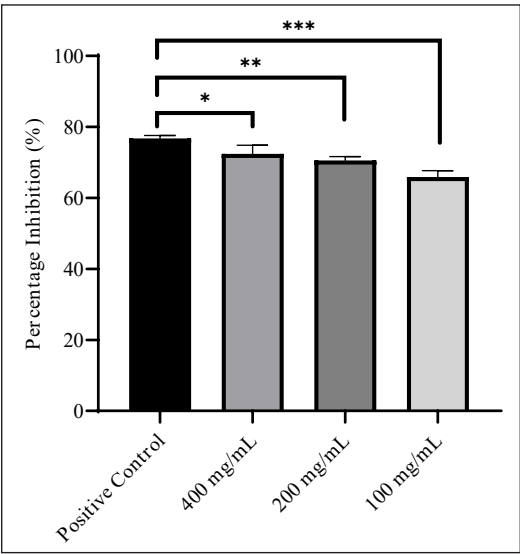


Figure 1. Bar chart representation for the percentage inhibition of COX-2 by different concentrations of EWT extract (400 mg/mL, 200 mg/mL, and 100 mg/mL) in comparison with celecoxib as a positive control. Error bars were indicated to the propagated standard errors of the mean. Data are expressed as Mean ± SD (n = 3) (*p<0.05, **p<0.001, and ***p<0.0001 versus positive control), while one-way ANOVA (analysis of variance) was performed, followed by Dunnett’s multiple comparison tests, indicating that they are statistically significant

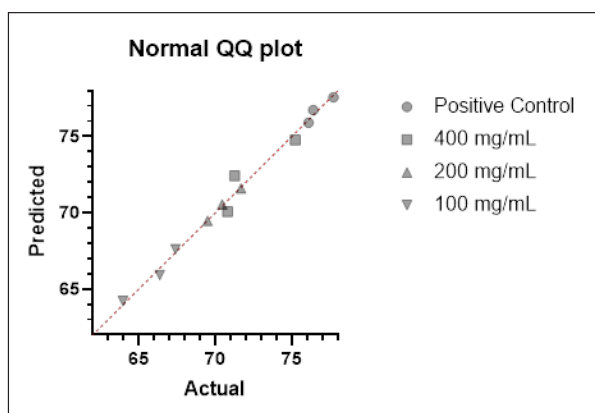


Figure 2. Normality and lognormality test (Normal QQ plot) of different concentrations of EWT extract (400 mg/mL, 200 mg/mL, and 100 mg/mL) tested against the COX-2 inhibition assay

At a concentration of 400 mg/mL, the EWT extract was found to exhibit a substantial inhibitory activity of 72.42%, which is comparable to that of celecoxib with 76.73% inhibition (Table 1). At 100 mg/mL of EWT extract that exhibited 65.91% of COX-2 inhibition, the IC_{50} of EWT extract can be approximated to be lower than 100 mg/mL. Both standard and EWT extracts exhibited apparent anti-inflammatory effects, where the performance exhibited by EWT highlights its potential as one of the natural alternatives. While celecoxib appears as a single compound with high potency, the observed slight difference in terms of inhibitory efficacy could be attributed to the complex composition of EWT that contains a blend of various strength bioactive compounds. However, the multifaceted nature of the extract could somehow provide additional therapeutic benefits, such as synergistic effects and or, reduced side effects, making it a promising candidate for further investigation. Similarly, at concentrations of 200 mg/mL and 100 mg/mL, the extract remained to exhibit notable inhibitory activity, with mean inhibition values of 70.53% and 65.91%, respectively. While these high concentrations demonstrate *in vitro* potential, they may not necessarily reflect *in vivo* bioavailability.

These findings suggest that EWT extract may contain bioactive compounds that are capable of inhibiting COX-2 activity, which appears to agree with its traditional use as an anti-inflammatory remedy. In addition, it aligns with the broader understanding of plant extracts that often contain bioactive compounds that can modulate inflammatory pathways. Knowing COX-2 is a key enzyme in the production of prostaglandins that mediates inflammation (Termer et al., 2021), the complex composition of plant extracts, including flavonoids and other secondary metabolites, can provide a multifunctional approach in reducing inflammation by disrupting various pathways involved in the inflammatory response (Attiq et al., 2018). This multi-targeted action may offer advantages over

single-compound drugs like celecoxib, by potentially reducing side effects and enhancing therapeutic efficacy via synergistic interactions exhibited by the bioactive compounds present in the extract.

Antioxidant Activity

In the FRAP assay, the reducing potential of the EWT extract was evaluated at varying concentrations, where a higher FRAP value corresponds to stronger antioxidant activity. For the DPPH and ABTS assays, the IC_{50} value representing the concentration of the sample required to inhibit 50% of free radical activity was determined, with a lower IC_{50} value indicating greater antioxidant potency (Irawan et al., 2022). Quercetin was used as a positive control, whereas methanol was used as a negative control in the FRAP assay. In the DPPH and ABTS assays, methanol is utilised as a vehicle control. It was analysed to rule out its effect towards radical scavenging activity. Since the solvent did not exhibit significant inhibition, the IC_{50} values of methanol were classified as 'Not Applicable' under DPPH and ABTS assays.

Generally, Table 2 demonstrated a moderate level of antioxidant activity of the EWT extract. In comparison to quercetin (positive control), the antioxidant potency of EWT extract was relatively lower. This observation could be attributed to the composition of the EWT that appears as a mixture of compounds, which are likely to contain a lower concentration of the specific compound(s) responsible for antioxidant activity (Spiegel et al., 2020). In the FRAP analysis, this trend was particularly apparent, where the $FeSO_4$ equivalent increased 11-fold as the EWT concentration increased 10-fold from 1 to 10 mg. In contrast to EWT, the standard quercetin exhibited only a 4.6-fold increase over the same concentration increment. We suggest that while the EWT extract possesses antioxidant capabilities, its activity could possibly be attributed to the concentration of active compounds within the mixture. In addition, while antioxidant activities exhibited by EWT were not as potent as the positive control, the presence of these active compounds could still contribute to the overall health benefits associated with the tonic.

While the EWT extract exhibited lower antioxidant activity compared to quercetin, its potential as an antioxidant source remains relevant and possesses antioxidant capability. The observed activity is indicative of the presence of active antioxidant compounds, even if at lower concentrations, due to the presence of numerous other constituents derived from the plant extracts in the formulation (Irawan et al., 2022).

Antimicrobial Activity

While the antimicrobial activity of EWT extract was evaluated with a disk diffusion method, susceptibility of the pathogens towards the EWT extract was assessed by measuring the clear zone (inhibition zone) surrounding the extract. Streptomycin sulfate was used as a

positive control for bacterial assay, whereas nystatin was used as a positive control for fungal assays. Table 3 shows the diameter of the inhibition zone of the EWT extract against the tested microorganisms. At the high concentration of 400 mg/mL of EWT extract, the antibacterial as well as antifungal capabilities are weaker in comparison with their respective standards, indicating the limited potential of EWT to serve as an antimicrobial agent (Table 3).

Table 2
Comparison among FRAP, DPPH and ABTS assays on EWT extract

No	Samples	FRAP (mg of FeSO ₄ equivalent/mg of extract)		DPPH IC ₅₀ (mg/mL)	ABTS IC ₅₀ (mg/mL)
		1 mg	10 mg		
1	EWT	0.14 ± 0.03	1.57 ± 0.32	2.0691 ± 0.21	10.0069 ± 0.51
2	Quercetin	5.34 ± 1.06	24.32 ± 5.76	0.0149 ± 0.00	0.1849 ± 0.03
3	Methanol	-0.01± 0.02	-0.01 ± 0.02	Not Applicable	Not Applicable

Note. All values represent means ± SD of three replicates; IC₅₀: Half maximal inhibitory concentration

Table 3
The inhibition diameter of EWT extract against pathogenic microbes

Microorganism	Inhibition Diameter (mm)		
	400 mg/mL of EWT Extract	Streptomycin Sulfate	Nystatin
Bacteria			
Bacillus subtilis	7.3 ± 0	35.0 ± 0	nt
Streptococcus pyogenes	12.0 ± 0	35.0 ± 0	nt
Fungi			
Candida albicans	6.0 ± 0	nt	25.0 ± 0
Aspergillus niger	8.0 ± 0	nt	20.0 ± 0

Note. * nt-not tested

While the EWT extract exhibited lower antioxidant activity compared to quercetin, its potential as an antioxidant source remains relevant and possesses antioxidant capability. The observed activity is indicative of the presence of active antioxidant compounds, even if at lower concentrations, due to the presence of numerous other constituents derived from the plant extracts in the formulation (Irawan et al., 2022).

CONCLUSION

Extracts from the EWT exhibit strong COX-2 inhibition, highlighting the tonic's potential for managing inflammatory conditions with notable antioxidants, and weak antimicrobial activities, supporting its traditional use for pain relief, reduction and management of inflammation. These results have demonstrated the potential of the waist tonic to serve as a natural remedy for conditions associated with oxidative stress and inflammation, although it was found to be less effective in microbial infections. In-depth investigation of the specific bioactive compounds isolated from EWT that are responsible for these effects, and moreover, the *in vivo* work, may enhance their application in modern healthcare.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

- Attiq, A., Jalil, J., Husain, K., & Ahmad, W. (2018). Raging the war against inflammation with natural products. *Frontiers in Pharmacology*, 9, Article 976. <https://doi.org/10.3389/fphar.2018.00976>
- Bennett, J. S., Daugherty, A., Herrington, D., Greenland, P., Roberts, H., & Taubert, K. A. (2005). The use of nonsteroidal anti-inflammatory drugs (NSAIDs). *Circulation*, 111(13), 1713-1716. <https://doi.org/10.1161/01.CIR.0000160005.90598.41>
- Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Analytical Biochemistry*, 239(1), 70-76. <https://doi.org/10.1006/abio.1996.0292>
- Daud, A., Hairon, S. M., & Shafei, M. N. (2023). Prevalence and risk factors of low back pain in Malaysia: A scoping review. *Malaysian Journal of Medical Sciences*, 30(3), 32-41. <https://doi.org/10.21315/mjms2023.30.3.3>
- Ferreira, M. L., de Luca, K., Haile, L. M., Steinmetz, J. D., Culbreth, G. T., Cross, M., Kopec, J. A., Ferreira, P. H., Blyth, F. M., Buchbinder, R., Hartvigsen, J., Wu, A. -M., Safiri, S., Woolf, A. D., Collins, G. S., Ong, K. L., Vollset, S. E., ... March, L. M. (2023). Global, regional, and national burden of low back pain, 1990-2020, its attributable risk factors, and projections to 2050: A systematic analysis of the Global Burden of Disease Study 2021. *The Lancet Rheumatology*, 5(6), e316-e329. [https://doi.org/10.1016/S2665-9913\(23\)00098-X](https://doi.org/10.1016/S2665-9913(23)00098-X)
- Fine, P. G. (2002). The role of rofecoxib, a cyclooxygenase-2-specific inhibitor, for the treatment of non-cancer pain: A review. *The Journal of Pain*, 3(4), 272-283. <https://doi.org/10.1054/jpai.2002.125957>

- Güllüce, M., Sökmen, M., Daferera, D., Agar, G., Özkan, H., Kartal, N., Polissiou, M., Sökmen, A., & Şahin, F. (2003). In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of *Satureja hortensis* L. *Journal of Agricultural and Food Chemistry*, 51(14), 3958-3965. <https://doi.org/10.1021/jf0340308>
- Hartvigsen, J., Hancock, M. J., Kongsted, A., Louw, Q., Ferreira, M. L., Genevay, S., Hoy, D., Karpinen, J., Pransky, G., Sieper, J., Smeets, R. J., & Underwood, M. (2018). What low back pain is and why we need to pay attention. *The Lancet*, 391(10137), 2356-2367. [https://doi.org/10.1016/S0140-6736\(18\)30480-X](https://doi.org/10.1016/S0140-6736(18)30480-X)
- Irawan, C., Dwi Putri, I., & Sukiman, M. (2022). Antioxidant activity of DPPH, CUPRAC, and FRAP methods, as well as activity of alpha-glucosidase inhibiting enzymes from *Tinospora crispa* (L.) stem ultrasonic extract. *Pharmacognosy Journal*, 14(5), 511-520. <https://doi.org/10.5530/pj.2022.14.128>
- Kuritzky, L., & Samraj, G. P. (2012). Nonsteroidal anti-inflammatory drugs in the treatment of low back pain. *Journal of Pain Research*, 5, 579-590. <https://doi.org/10.2147/JPR.S6775>
- Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Reviews*, 4(8), 118-126. <https://doi.org/10.4103/0973-7847.70902>
- Muniandy, R. K., Mudin, K., Yeap, B. T., & Voo, S. K. (2022). Increased incidence and aetiological factors of back pain among Universiti Malaysia Sabah staff and undergraduates during the COVID-19 lockdown period. *Medical Journal of Malaysia*, 77(2), 185-188.
- National Pharmaceutical Regulatory Agency. (n.d.). *Ecolite® Waist Tonic (Product Search)*. Quest 3 Plus. <https://quest3plus.bpfk.gov.my/pmo2/detail.php?type=product&id=MAL21096100T>
- Sivasampu, S., Yvonne, L., Norazida, A. R., Hwong, W. Y., Goh, P. P., & Hisham, A. N. (2014). *National Medical Care Statistics (NMCS) 2012*. National Clinical Research Centre. <https://doi.org/10.13140/RG.2.1.3663.5761>
- Spiegel, M., Kapusta, K., Kołodziejczyk, W., Saloni, J., Żbikowska, B., & Hill, G. A. (2020). Antioxidant activity of selected phenolic acids-feric reducing antioxidant power assay and QSAR analysis of the structural features. *Molecules*, 25(13), Article 3088. <https://doi.org/10.3390/molecules25133088>
- Stratil, P., Klejdus, B., & Kubáň, V. (2006). Determination of total content of phenolic compounds and their antioxidant activity in vegetables—Evaluation of spectrophotometric methods. *Journal of Agricultural and Food Chemistry*, 54(3), 607-616. <https://doi.org/10.1021/jf052334j>
- Strine, T. W., & Hootman, J. M. (2007). U.S. national prevalence and correlates of low back and neck pain among adults. *Arthritis & Rheumatism*, 57(4), 656-665. <https://doi.org/10.1002/art.22684>
- Termer, M., Carola, C., Salazar, A., Keck, C. M., Hemberger, J., & von Hagen, J. (2021). Activity-guided characterisation of COX-2 inhibitory compounds in *Waltheria indica* L. extracts. *Molecules*, 26(23), Article 7240. <https://doi.org/10.3390/molecules26237240>
- van der Gaag, W. H., Roelofs, P. D., Enthoven, W. T., van Tulder, M. W., & Koes, B. W. (2020). Non-steroidal anti-inflammatory drugs for acute low back pain. *Cochrane Database of Systematic Reviews*, 2020(4), Article CD013581. <https://doi.org/10.1002/14651858.CD013581>

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