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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 × 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 TRB10%	T2 TRB15%	T3 TRB20%	T4 WDZ 10%	T5 WDZ 15%	T6 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 TRB 10%	T2 TRB 15%	T3 TRB 20%	T4 WDZ 10%	T5 WDZ 15%	T6 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 $CaCl_2 \cdot 2H_2O$, 16.676g of $MnCl_2 \cdot 4H_2O$, 1.67g of $CoCl_3 \cdot 6H_2O$, and 13.33g of $FeCl_3 \cdot 6H_2O$ 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 $MgSO_4 \cdot 7H_2O$ 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 $Na_2S \cdot 7H_2O$ 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 CO_2. The filtrate obtained was subsequently combined with an additional 200 ml of medium and homogenised for 20 seconds under continuous CO_2 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 CO_2 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 H_2O 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).

$$\text{IVOMD (\%)} = 14.88 + 0.889GP + 0.45CP + 0.0651XA \quad [2]$$

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:

$$\text{ME (MJ/kg DM)} = 1.06 + 0.157GP + 0.084CP + 0.22CF - 0.081CA \quad [3]$$

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							
	T1 TRB 10%	T2 TRB 15%	T3 TRB 20%	T4 WDZ 10%	T5 WDZ 15%	T6 WDZ 20%	T7 CON	p-value
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^{e} ±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^{e} ±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 10%	49.57^{ab} ±2.94	5.69^{a} ±0.01	10.78^{a} ±0.46	64.97^{b} ±0.54	72.68^{ab} ±2.62
T5 WDZ 15%	47.66^{ab} ±3.64	5.58^{b} ±0.04	10.51^{ab} ±0.57	61.73^{c} ±0.57	68.82^{bc} ±3.23
T6 WDZ 20%	45.54^{bc} ±4.46	5.51^{bc} ±0.01	9.81^{ab} ±0.70	60.89^{c} ±0.56	68.63^{bc} ±3.97
T7 CON	60.22^{a} ±7.39	5.72^{a} ±0.02	11.79^{a} ±1.16	73.58^{a} ±0.57	81.71^{a} ±6.09
p-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)	8.48^{a} ±1.75	6.93^{ab} ±0.45	5.42^{b} ±0.75	5.56^{b} ±1.23	6.32^{ab} ±0.60	7.44^{ab} ±1.24	7.51^{ab} ±0.48	*
b (ml)	70.16^{a} ±6.70	51.29^{b} ±3.29	49.36^{b} ±3.15	50.98^{b} ±4.10	57.39^{b} ±1.55	57.61^{b} ±1.66	54.70^{b} ±0.65	**
c (ml/h)	0.07^{a} ±0.007	0.07^{a} ±0.006	0.08^{a} ±0.018	0.09^{a} ±0.022	0.08^{a} ±0.006	0.08^{a} ±0.005	0.09^{a} ±0.011	NS
a + b (ml)	76.49^{a} ±7.55	58.22^{bc} ±3.17	54.78^{c} ±3.84	56.54^{bc} ±5.33	65.87^{ab} ±1.44	65.05^{abc} ±1.79	62.21^{bc} ±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

byGaviria-Uribe 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 WDZ-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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