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16S rRNA-based Metagenomic Analysis of Beeswax-coated Saba Banana (*Musa* × *paradisiaca*) Pseudostem

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ABSTRACT

Bananas are one of the most popular fruits, and their production generates significant agricultural waste. Banana pseudostems, a by-product of the banana industry, are being investigated as a renewable and biodegradable alternative to synthetic food packaging materials. However, these pseudostems have the potential to harbor harmful bacteria due to their natural fiber composition. Therefore, this study analyzes the effect of beeswax coating on the microbial communities in banana pseudostems. The microbial community is analyzed through a metagenomics approach that targets the 16S rRNA gene of the Saba banana (*Musa* × *paradisiaca*) pseudostem. Two experimental conditions were considered: pseudostem with beeswax coating and pseudostem without beeswax coating. The findings indicate that the microbial communities in all samples are primarily composed of the phyla Proteobacteria, Cyanobacteria, and Firmicutes. The dominant species found in uncoated banana pseudostem is *Pantoea* sp. At-9b, *Escherichia coli*, *Synechococcus* sp. JA-3-3-Ab, *Pantoea vagans*, and *Klebsiella pneumoniae*. The dominant species found in beeswax-coated banana pseudostem is *Synechococcus* sp. JA-3-3-Ab, *Pseudanabaena* sp. PCC 7367, *Chroococcidiopsis thermalis*, *Priestia megaterium*, and *Ammonifex degensii*. The Chao1, Shannon, Simpson, and Equitability indices indicate that the species richness, diversity, and evenness in the uncoated banana pseudostem are higher than in the

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beeswax-coated banana pseudostem. The degree of similarity between bacterial populations found in uncoated banana pseudostem and beeswax-coated banana pseudostem is around 53.9%.

Keywords: 16S rRNA, banana pseudostem, beeswax, metagenomics

INTRODUCTION

Bananas are extensively grown and are recognized as one of the most widely produced fruits globally. They also play a crucial role as an edible fruit in Indonesia, where various banana studies have been carried out (Dwivany et al., 2014, 2016, 2021; Khairiya et al., 2023). Based on data from the Central Statistics Agency, banana production in Indonesia alone reached 8.74 million tons in 2021 (Badan Pusat Statistik [BSP], 2024). Bananas are perennial plants that have unique characteristics. The crops are harvested singularly and have a brief life cycle lasting one year. When the fruits are harvested, the whole plant must be cut down (Padam et al., 2014; Saraiva et al., 2012). Bananas produce by-products such as rachis, stems, and banana leaves during production (Acevedo et al., 2021). These by-products, which are non-fruit biomass, contribute to a large amount of waste (Saraiva et al., 2012). On average, the waste produced by a single banana plant can reach 80% of its total mass (Padam et al., 2014). Thus, it can be estimated that banana waste produced in Indonesia can reach several tons. Waste produced from bananas can cause various problems in banana production areas, including the growth of banana pathogen deposits and pests (Yoga Milani et al., 2020). The customary practice in banana plantations is to allow banana stems and leaves to undergo decomposition, enriching the soil with nutrients.

However, this practice can interfere with farmers harvesting fruits (Padam et al., 2014). Moreover, after decomposition, banana waste can produce harmful gases like hydrogen sulfide and ammonia (Saraiva et al., 2012). In some cases, open-fire burning is still practiced in disposing of banana waste, further contributing to environmental problems (Padam et al., 2014). Efforts have been made to process banana waste into value-added products to address this issue. Banana stem waste has been utilized to make fertilizer (Padam et al., 2014), animal feed (Yanuartono et al., 2020), paper, and craft materials (Saraiva et al., 2012). Additionally, banana stems have the potential to be an alternative to disposable synthetic packaging, such as styrofoam (Yoga Milani et al., 2020). However, using banana stems as food packaging poses challenges due to their susceptibility to microbial growth in the surface area, leading to odor, discoloration, and potential infection from pathogenic bacteria (Yoga Milani et al., 2020). Beeswax has emerged as a popular coating for natural ingredients, including banana stems, to overcome these challenges. Beeswax possesses antimicrobial properties (Beck et al., 2021), hydrophobic properties, and reduced water vapor permeability (Trevisani et al.,

2017), making it an ideal coating for food packaging. It is also considered generally recognized as safe (GRAS) by the United States Food and Drug Administration (Szulc et al., 2020). Previous studies have shown the potential of using beeswax as a coating for cotton fabrics for packaging purposes (Beck et al., 2021; Pinto et al., 2017). Based on the documentation of its properties, beeswax has the potential to be applied to banana stems and suppress microbial growth. In addition, beeswax can also answer the urgency of the material with a biodegradable, renewable, and non-toxic hydrophobic surface to replace synthetic packaging (Asim et al., 2022).

However, no studies have examined the effect of beeswax coating on the bacterial community of banana stems used as food packaging. Therefore, this study aims to analyze the differences in composition, the diversity index, and the similarity in the microbial communities found in banana stems that are coated and not coated with beeswax.

MATERIALS AND METHODS

Sample Preparation

The sample used in this study was a sample of Saba banana (*Musa* × *paradisiaca*) sourced from the Sumedang region in West Java. Parties from Bandung National Institute of Technology prepared the sample. The part of the Saba banana used is the outermost fourth layer of the pseudostem of the banana. The sample is subjected to two treatments: banana pseudostem samples coated with beeswax and uncoated. The diagram of the samples used in this study is presented in Figure 1.

The beeswax used is obtained from BioPolish and possesses a food-grade certification. The pseudo-stem of the Saba banana is initially dried under the sun. The pseudo-stems that are not too large are then selected and prepared. The pseudo-stems are cut into two equal-sized pieces measuring 4 cm × 4 cm each. A single segment of the pseudo-stem was left untreated to serve as a control sample. Subsequently, the other segment of the pseudo-stem was coated with beeswax thrice within 48 hr to serve as the treated sample. The visual representations of the samples are depicted in Figure 2.

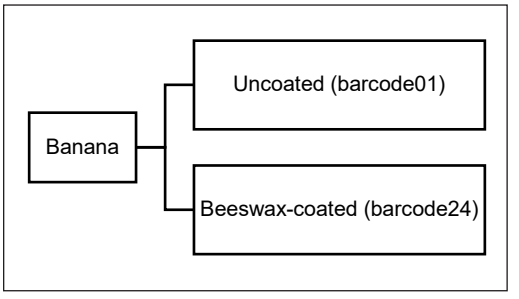
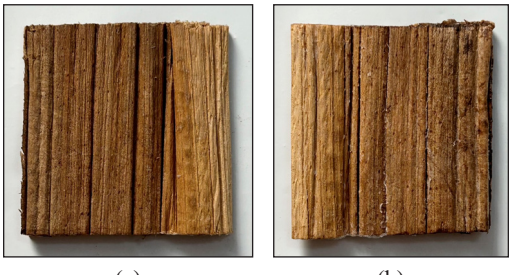


Figure 1. Sample diagram used in this study



(a) (b)

Figure 2. Banana pseudostem samples: (a) uncoated; and (b) beeswax-coated

Metagenomic Analysis

The genomic DNA from each banana pseudostem sample was isolated using Genomic DNA Mini Kit Plant (Geneaid Biotech Ltd., Taiwan) (Karmawan et al., 2009). The concentration of DNA was assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a Qubit fluorometer (Thermo Fisher Scientific, USA). Library preparation is done utilizing kits provided by Oxford Nanopore Technology (ONT, United Kingdom). The sequencing process is conducted with GridION (ONT, United Kingdom) and operated with MinKNOW software (version 20.06.9). After sequencing, base calling was done using Guppy (version 4.0.11) in high accuration mode (Wick et al., 2019). After obtaining the sequencing results in the form of FASTQ files, the quality of the FASTQ files is visualized with NanoPlot (version 1.41.3) (De Coster et al., 2018).

This study used taxonomic annotations to determine the bacteria in bananas with and without beeswax coating. Classification of reads is carried out with Centrifuge (version 1.0.3). Centrifuge is a fast and sensitive classifier for microbial sequences with low memory requirements but has speeds comparable to the fastest systems (D. Kim et al., 2016). The index used to classify the samples in this study is the index of bacteria and archaea downloaded from the Centrifuge website (https://ccb.jhu.edu/software/centrifuge). The command used in the Centrifuge can also be seen on the Centrifuge website (https://ccb.jhu.edu/software/centrifuge).

After reading the classification, relative abundance is visualized with KronaTools (https://github.com/marbl/Krona). In addition, diversity analysis and data visualization were also carried out with RStudio (version 2022.12.0).

RESULTS AND DISCUSSION

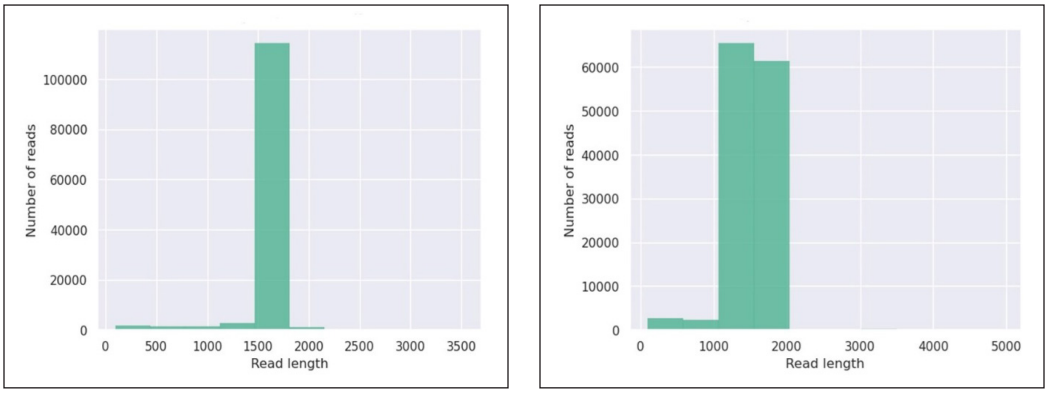
Sequence Statistics

The sequence statistics of the samples are presented in Table 1.

The total base of the uncoated sample was 188,926,678 bases, whereas the beeswax-coated sample was 202,090,361 bases. The total amplicon reads obtained were 122,874 for the uncoated sample and 131,995 for the beeswax-coated sample. The distribution of reading length is presented in Figure 3. The average read length for the uncoated sample was 1,574.6 bp, and for the beeswax-coated sample, it was 1,531.0 bp. The average read quality for both the uncoated and beeswax-coated samples was 11.

Table 1
Banana stem sample sequencing statistics

No.	Sample	Number of reads	Length (bp)	Quality score	Total bases
			mean	mean	
1.	Uncoated sample (barcode01)	122,874	1,574.6	11	188,926,678
2.	Beeswax-coated sample (barcode24)	131,995	1,531.0	11	202,090,361



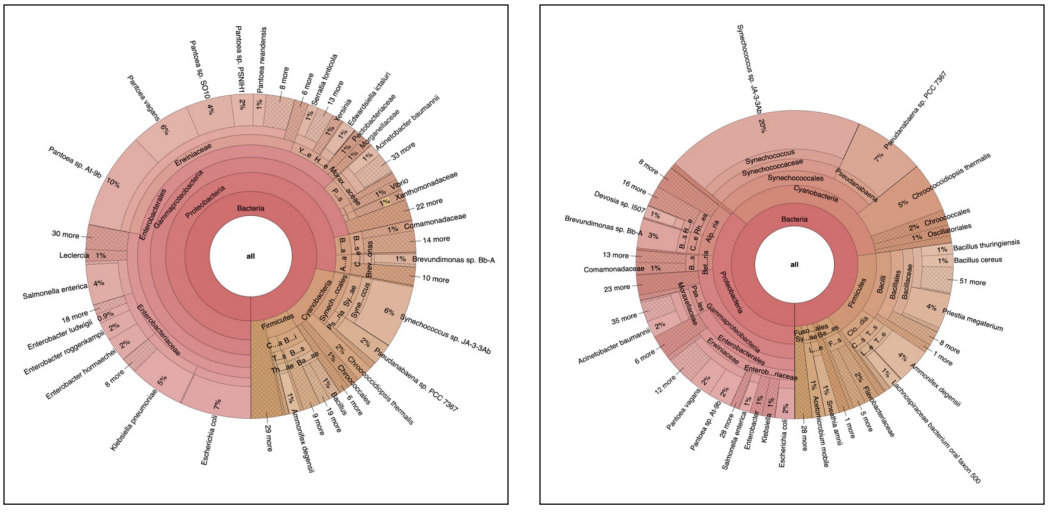
(a) (b)

Figure 3. Distribution of read lengths: (a) Uncoated banana pseudostem sample; and (b) beeswax-coated banana pseudostem sample

Microbial Community Structure

The reads obtained from sequencing, both uncoated and beeswax-coated samples, can be categorized based on taxonomic classification, ranging from the domain level to the species level. The taxonomic annotation results are then represented using a Krona chart, which effectively displays the comparative prevalence of the two samples across various taxonomic levels. Figure 4 displays a graphic representation of the taxonomic composition observed in the two samples.

At the phylum level, the predominant bacterial taxa identified in both samples, namely uncoated banana pseudostem and beeswax-coated, were Proteobacteria, Cyanobacteria, and Firmicutes, but with



(a) (b)

Figure 4. Krona chart: (a) Uncoated banana pseudostem sample; and (b) beeswax-coated banana pseudostem sample

varying proportions. Proteobacteria are the most extensive taxonomic group within the bacterial domain. Numerous human pathogens, such as *Brucella*, *Rickettsia*, *Escherichia*, *Salmonella*, and *Helicobacter*, were also found in the Proteobacteria phylum, encompassing human diseases (Rizzatti et al., 2017). Most organisms belonging to the phylum Proteobacteria exist in a free-living state, which includes a diverse range of nitrogen-fixing bacteria (Sharmin et al., 2013). Firmicutes, which encompasses the genus *Bacillus*, have a significant role within the plant microbiome (Borriss, 2020). Cyanobacteria, regarded as one of the most ancient organisms on the planet, exhibit a remarkable ability to thrive in diverse environments, spanning from arid deserts to scorching hot springs and even arctic regions. Cyanobacteria possess diverse capabilities, including establishing biofilms to protect in diverse environmental circumstances (Kollmen & Strieth, 2022). Furthermore, Cyanobacteria possess a remarkable capacity to fix atmospheric nitrogen and exhibit competitiveness within microflora communities. Cyanobacteria exhibit beneficial biological functions, including their efficacy as biocidal agents against bacteria, fungi, and nematodes, as demonstrated (Elagamey et al., 2023).

The relative abundances of both samples at the phylum level are depicted in Figure 5. Within the uncoated sample, 78% of the total reads are attributed to Proteobacteria. Additionally, the phylum Cyanobacteria comprises 13% of the sample. The phylum Firmicutes comprises 6% of the total reads in the sample. In the beeswax-coated sample, the phylum Proteobacteria comprises 36% of the total reads in the sample. Similarly, Cyanobacteria represents 36% of the total reads in the sample. The phylum Firmicutes comprises 18% of the total reads in the sample.

Other studies in the phyllosphere and other plant species have also reported the dominant phylum found in banana pseudostems. The bacteria identified in banana plants are into four primary phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Additionally, there are several minor phyla, including Cyanobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes, Acidobacteria, and Spirochaetes (Beltran-Garcia et al., 2021). Actinobacteria, Bacteroidetes, Firmicutes, and especially Proteobacteria are often found in bacterial communities on the surface of plants above the ground (phyllosphere). The main phylum found on banana stems can also be found in other plant species, such as rice,

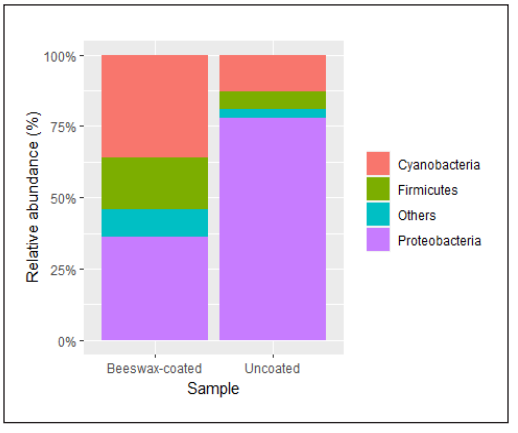


Figure 5. Relative abundance of the four dominant phyla in uncoated and beeswax-coated samples

mustard, spinach, and 56 other tree species (Bulgarelli et al., 2013). In addition to Firmicutes and Proteobacteria (alpha-, beta-, gammaproteobacteria), Cyanobacteria are often found in the phyllosphere environment (Kaewkla & Franco, 2013). Based on a study conducted by Peng et al. (2022), dragon fruit plant stems are also dominated by phyla Proteobacteria and Firmicutes bacteria.

At lower taxonomic levels, differences were also found in samples of banana stems coated with beeswax and not coated with beeswax. Proteobacteria members have the highest relative abundance for samples without beeswax coating, while Cyanobacteria members have the highest relative abundance for beeswaxed banana stems. For the specific genus level in banana stem samples not coated with beeswax, the genus with the highest relative abundance was *Pantoea* (26%). In comparison, other genera had a relative abundance of less than 10%. For the specific genus level in banana stem samples not coated with beeswax, the genus with the highest relative abundance was *Synechococcus* (20%). In comparison, other genera had a relative abundance of less than 10%.

At the species level, there are significant differences in the species that dominate the two samples (Table 2). In the uncoated sample, the five species that exhibit the highest relative abundance were *Pantoea* sp. At-9b (10%), *E. coli* (7%), *Synechococcus* sp. JA-3-3-Ab (6%), *P. vagans* (6%), and *K. pneumoniae* (5%), respectively. In the beeswax-coated sample, the five species that exhibited the highest relative abundance were *Synechococcus* sp. JA-3-3-Ab (20%), *Pseudanabaena* sp. PCC 7367 (7%), *C. thermalis* (5%), *P. megaterium* (4%), and *A. degensii* (4%).

An interesting phenomenon in this study is the difference in bacteria dominating banana stems in both samples at each taxonomic level (Figure 4). This disparity is particularly evident at the species level, where the predominant bacteria found in the uncoated sample had a low relative abundance in the beeswax-coated sample.

Some dominant species in the uncoated sample are classified as opportunistic pathogens, including various species in the genus *Pantoea*, which can cause several infections, although at much lower rates than *E. coli* and *K. pneumoniae* (Cunningham & Leber, 2018). *Klebsiella pneumoniae*, in

Table 2
Species with the highest relative abundance in uncoated and beeswax-coated banana pseudostem samples

Uncoated banana pseudostem samples		Beeswax-coated banana pseudostem samples	
Name	**Relative abundance (%)**	**Name**	**Relative abundance (%)**
Pantoea sp. AT-9b	10	*Synechococcus* sp. JA-3-3-Ab	20
Eschericia coli	7	*Pseudanabaena* sp. PCC 7367	7
Synechococcus sp. JA-3-3-Ab	6	*Chroococcidiopsis thermalis*	5
Pantoea vagans	6	*Priestia megaterium*	4
Klebsiella pneumoniae	5	*Ammonifex degensii*	4

general, is a human pathogen that can cause pneumonia and various infections in the human body (Borkar & Ajayasree, 2021). *Escherichia coli* is also a bacterium involved in various infections, including digestive ones (Jnani & Ray, 2024). Consequently, the application of beeswax coating has the capacity to reduce the presence of pathogenic germs.

This discovery is in line with prior studies conducted in the field. One possible explanation for this phenomenon is attributed to the antimicrobial properties inherent in beeswax, whether independently or in conjunction with other natural substances. Crude beeswax has antimicrobial properties against Gram positive bacteria, such as *Staphylococcus aureus*, *Streptococcus epidermidis*, *Streptococcus pyogenes*, and Gram negative, including *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Aspergillus niger*, and *E. coli* (Fratini et al., 2016). Beeswax has also been found to be antimicrobial against bacteria of the genera *Bacillus*, *Escherichia*, *Listeria*, *Proteus*, *Pseudomonas*, *Salmonella*, *Staphylococcu*s, and various genera of fungi (Szulc et al., 2020). Beck et al. (2021) have also documented the laboratory-based antibacterial properties of beeswax when applied to cotton packing materials.

While the precise mechanism underlying the antibacterial activity of beeswax has not been fully explored, several constituents of beeswax have been documented to possess antimicrobial properties. These include polyphenol components (Anilakumar et al., 2007) and fatty acids (Desbois & Smith, 2010; Fratini et al., 2016). Fatty acids are known to be promising antibacterial agents because they can destabilize bacterial cell membranes. This membrane-destabilizing activity can lead to increased cell permeability and cell lysis. It can inhibit bacterial cell growth (bacteriostatic) or cell death (bactericidal) (Yoon et al., 2018). Additionally, oleic acid, a specific type of fatty acid, can enhance membrane permeability as measured by polarized fluorimetry (Chamberlain et al., 1991). A decreased polarization value indicates an increase in membrane fluidity caused by oleic acid, resulting in cell death. Polyphenols have also been reported to have antimicrobial activity against various types of bacteria. A possible mechanism of action is the aggregatory effect on bacterial cells (Cushnie & Lamb, 2011; Daglia, 2012).

Another phenomenon in this study revealed that the five species that showed comparatively lower abundance in the uncoated sample had higher relative abundance in banana stems treated with beeswax coating. This phenomenon can occur due to the resistance of some species to beeswax. The resilience observed in plants can be attributed to the fertilizers used in the soil, which subsequently move to the pseudostem area, facilitated by precipitation events. Rossmann et al. (2012) have also documented fertilizers derived from animal excrement as a source of antibiotic resistance in bacteria. This microbial community composition data suggests that applying beeswax coating influences the composition of the microbial community

on banana pseudostems. Nevertheless, the bacteria identified as prevalent in the beeswax-coated samples are not categorized as pathogenic microorganisms.

Diversity Estimation

Diversity is one of the most frequently used components in community characterization. Diversity at the habitat level includes species richness and evenness. The species richness index calculates the number of individuals per unit area or sample. In contrast, the evenness index assesses the proportional representation of various species in a community, resulting in a visible distribution. A community with diverse species and equal individuals would exhibit a high evenness index value. In contrast, a community dominated by a particular species in terms of individual count would have a low evenness index value (Thukral, 2017). Alpha diversity is the variability of species in a single sample (Calle, 2019). Alpha diversity can encapsulate the structure of a community depending on its abundance, evenness, or both. In microbial ecology, alpha diversity can determine the difference between environments (Willis, 2019). The alpha diversity indices used in this study include Chao1, Shannon, Simpson, and Equitability, which can be seen in Figure 6.

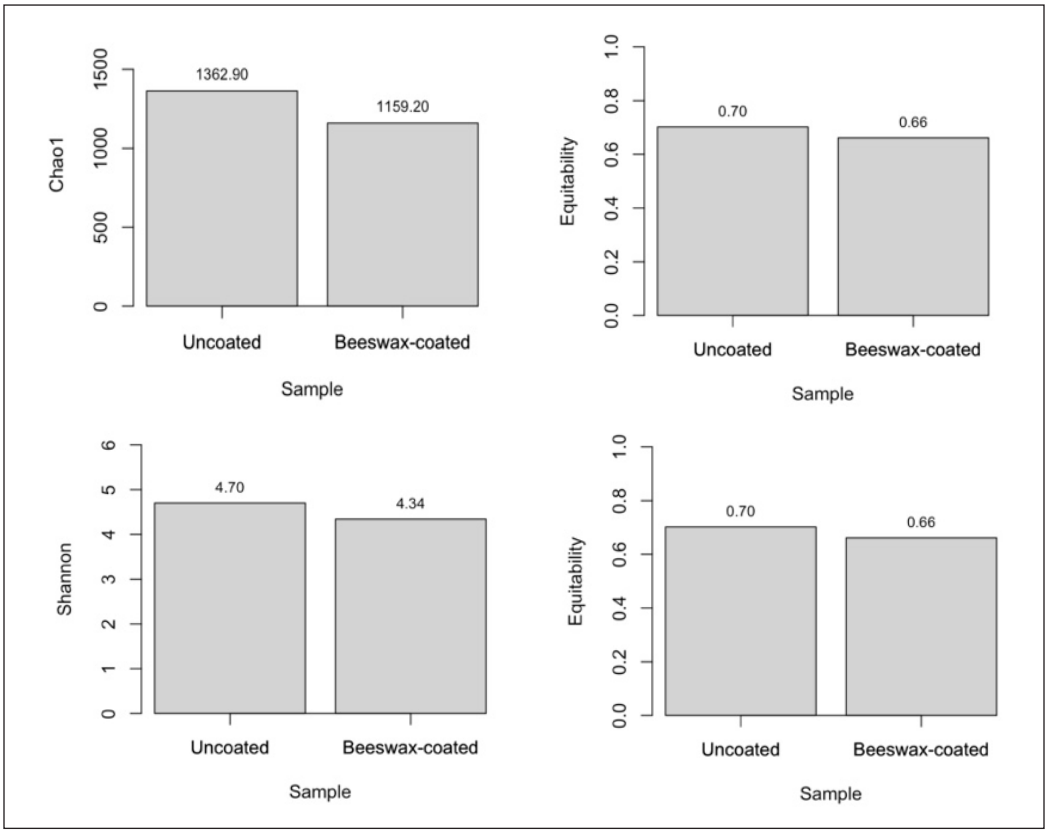


Figure 6. Alpha diversity index of banana stem samples coated and not coated by beeswax

The Chao1 index is a quantitative measure utilized to quantify species richness by considering the relative abundance of different species within a given ecological community. The Shannon and Simpson indices are quantitative measures that can offer insights into the composition of a community and describe population diversity in a sample. Both indices assess diversity by considering both species richness and species evenness. However, their calculations differ in the weight assigned to these two factors. The Shannon index estimates diversity by assigning greater importance to species richness, whereas the Simpson index places greater emphasis on species evenness (B.-R. Kim et al., 2017; Simpson, 1949). The Equitability index is an index that measures the evenness of species in the number of individuals of each species (Thukral, 2017).

Based on the alpha diversity indices in Figure 6, the two samples generally have notable differences. The Chao1 index shows that the uncoated sample has a higher species richness than the beeswax-coated sample. Shannon and Simpson diversity indices show that the uncoated sample is more diverse than the beeswax-coated sample. Based on Shannon index criteria in Ulfah et al. (2019), the Shannon index scores for both samples indicate a high level of diversity ($H' \geq 3$). According to the Simpson index, as the index score approaches 1, the level of diversity increases (Sagar & Sharma, 2012). Based on Simpson index criteria on Wahyuningsih et al. (2019), the Simpson index scores for both samples also indicate a high level of diversity ($0.60 < D \leq 1$). Furthermore, the Equitability index shows that the uncoated sample has a higher species evenness than the beeswax-coated sample. Based on the range of the Equitability index scores from Krebs (1989), both samples are categorized as unstable communities ($0.5 < E \leq 0.75$).

Microbial Community Similarities

Figure 7 depicts a Venn diagram illustrating the similarity of bacterial communities in banana stem samples, comparing the uncoated and beeswax-coated samples.

According to 16S rRNA gene sequencing data, 2,218 operational taxonomic units (OTUs) in the uncoated and beeswax-coated samples were detected overall. The observed similarity between the two samples was 1,196 OTUs (53.9%).

In the uncoated sample, 497 unique OTUs were found unique to the uncoated sample and were not found in the beeswax-coated sample. This number can indicate

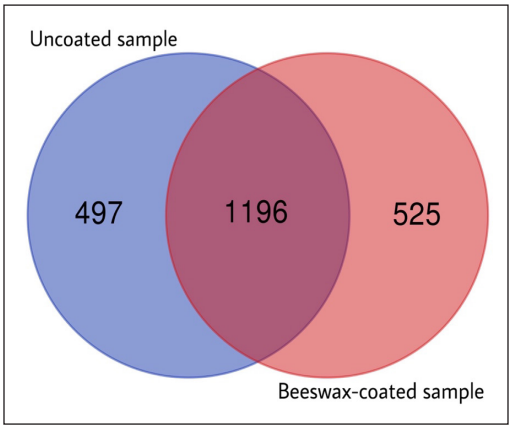


Figure 7. Venn diagram of the uncoated banana pseudostem samples (barcode01) and beeswax-coated banana pseudostem samples (barcode24)

the amount of OTUs lost in banana stems due to beeswax coating. As previously reported, beeswax poses antimicrobial activity due to its composition, including polyphenols (Anilakumar et al., 2007), fatty acids (Desbois & Smith, 2010; Fratini et al., 2016), and propolis (Pinto et al., 2017). Moreover, beeswax has physicochemical properties that can affect the microbial community (Zhang et al., 2023). Beeswax has also been reported to provide a barrier to oxygen, light, and water vapor, potentially impacting microbial activity in water banana stems (Trevisani et al., 2017).

Species uniqueness was also found in the beeswax-coated sample. There were 525 OTUs unique to the beeswax-coated sample that were not found in the uncoated sample. This number can indicate the number of OTUs coming from the beeswax itself. However, it is worth noting that the dominant members of the OTU detected (Table 2) are not pathogenic. Furthermore, these identified OTUs have the potential to exhibit antagonistic activities toward pathogens. As microbial communities in an environment can form interactive networks, this interaction can also occur among fellow species, including antagonistic ones (Hibbing et al., 2010). Therefore, the microbes coming from the beeswax itself may have antagonistic activities toward other bacteria.

CONCLUSION

The research shows that the microbial communities in the uncoated banana pseudostem samples and the beeswax-coated banana pseudostem samples are very different at the species level. The five most dominant species in the uncoated sample are *Panitoea* sp. At-9b, *E. coli*, *Synechococcuis* sp. JA-3-3-Ab, *P. vagans*, and *K. pneumoniae*. The five most dominant species in the beeswax-coated sample are *Synechococcus* sp. JA-3-3-Ab, *Pseudanabaena* sp. PCC 7367, *C. thermalis*, *P. megaterium*, and *A. degensii*. The Chao1, Shannon, Simpson, and Equitability indices for the uncoated sample were 1,362.90; 4.70; 0.97; 0.70, respectively, and for the beeswax-coated sample, they were 1,159.20; 4.34; 0.94; 0.66, respectively. Furthermore, the uncoated sample has higher species richness, diversity, and evenness than the beeswax-coated sample. The similarity of bacterial communities in the beeswax-coated and uncoated samples was 53.9%.

For further research, it is recommended that further examinations be conducted on the bacterial composition of banana stems, comparing those that are coated with beeswax and those that are not. Furthermore, beeswax can be combined with other naturally occurring substances with antimicrobial properties to enhance its antibacterial efficacy, thereby serving as a coating agent.

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