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Analysis of Bacterial Communities and Physico-chemical Properties of Grain Corn Silage Using 16S Amplicon Metagenomics in Malaysia

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ABSTRACT

Tropical regions produce silage that is susceptible to spoiling due to excessive humidity and warmth. Consequently, identifying native bacteria as a possible inoculant is important to enhancing the quality of silage. The aim of this work was to use amplicon metagenomics to identify the bacterial community and functional changes related to ensiling and to forecast possible bacterial inoculant related to grain corn silage quality in the Malaysian climate. The fermentation characteristics and functional bacterial populations grain corn were produced and studied. After fermentation, the grain corn silage had a lactic acid bacteria (LAB) predominance. The dominant taxa in fresh grain corn, *Leuconostoc* & *Pseudomonas* were displaced by LAB, namely *Weissella* and *Lactobacillus* and showed high silage quality with an increase in lactic acid (LA) and acetic acid (AA), conversely decrease in water-soluble carbohydrates (WC). Tax4fun's functional prediction revealed metabolic pathways of coenzyme and transport and metabolism were depleted while synthesis of secondary metabolites which associated to fermentation activities ($p<0.05$) was enriched in after ensiling, likely to support bacterial growth during silage fermentation and produce metabolic byproducts like lactic acid. This study highlighted the presence and potential roles of homolactic and heterolactic bacterial populations before and after ensiling, which can be utilized to produce more effective bacterial additives for improving the fermentation quality of grain corn silage in tropical climates like Malaysia.

1. INTRODUCTION

Malaysia's livestock industry is crucial for the country's economy, where the total trade of livestock valued at RM1.4 billion in 2023 [1]. However, the industry struggles to maintain a consistent and high-quality feed supply throughout the year due to seasonal variability, inconsistent nutrient content, and feed spoilage [2]. Tropical climates support year-round growth, however, the Malaysian climate experiences drought and monsoon season when the availability of fresh forage is limited. Silage allows for more flexible feeding management where it can preserve the fresh forage to be fed to the ruminants during those seasons. Farmers can store large quantities of feed and use it as needed, rather than relying on the availability of fresh forage. Therefore, stabilising feed availability and quality through silage can contribute to more predictable and stable production outcomes, which can have economic benefits for farmers.

In Malaysia, the most used silage is derived from tropical forage crops such as corn and Napier which are well-suited to the climate and conditions of the region. Corn (maize) is one of the most popular crops for silage in Malaysia due to its high energy content and digestibility [3]. Corn silage is highly

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valued for dairy and beef cattle due to its high energy content as it provides a good balance of carbohydrates and fibre [4]. Grain corn typically has a higher dry matter content compared to sweet corn [4]. This is important for silage fermentation and preservation. Ideal silage should have around 30-35% dry matter to ensure proper fermentation and prevent spoilage [5].

Grain corn has a higher starch content, which provides more energy to the livestock corn [3]. Starch is a crucial component for high-quality silage as it improves the energy density of the feed, benefiting the growth and milk production of cattle. Sweet corn, on the other hand, is typically grown for human consumption and has a higher sugar content but lower overall dry matter and starch content compared to grain corn. In addition, the structure of sweet corn plants and their lower yield makes them less suitable for large-scale silage production [3]. Therefore, grain corn is more commonly used and suitable for making high-quality silage due to its higher dry matter and starch content, greater yield, better digestibility, and more substantial plant structure.

Silage can be prepared by using a variety of crops, whether combining a different type of forage or only using one type of forage. Ensiling is a process of fermenting the forage into silage which involves a variety of microbial communities, with the epiphytic microbial communities of fresh forages playing a key role in ensuring a good silage quality being produced. Microbial fermentation during the ensiling generates a variety of metabolites that can alter the nutritional value of forage [6]. Furthermore, through ensiling, the energy content of the crops is preserved while maintaining a high nutritional value to be used as ruminant feed. The anaerobic condition during the ensiling process facilitates the growth of lactic acid bacteria (LAB). During carbohydrate fermentation, LAB creates lactic acid (LA) and lowers pH, which stops spoilage bacteria from growing and stabilises the silage. Even though there is a concerted effort to manufacture silage in Malaysia, tropical nations like Malaysia produce low-quality silage that spoils quickly [7]. This is because unfavourable bacteria thrive in tropical climates with high humidity [8, 9, 10]. However, compared to other nations like China, Malaysia lacks information on the indigenous microbial populations engaged in the ensiling process, which makes it difficult to choose the right additives to raise the product's quality.

Research on the bacterial communities involved in corn silage ensiling is limited. The most prevalent genera of LAB, which are frequently involved in ensiling are *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Enterococcus*, and *Streptococcus* [11]. Moreover, several of these taxa are common in tropical grass silages [12]. Silage fermentation is often carried out in a bunker silo or silo drum in temperate countries. To improve the quality of the silage, a bacterial additive is added [13, 14, 15]. Bacterial additions have been utilized to enhance the nutritional quality and ensiling practices of silage since the 1990s [11]. This has resulted in a decrease in dry matter losses and an increase in aerobic stability. In addition, according to Muck et al. (2018) [20], LAB is the most widely utilized bacterial additive to enhance silage fermentation. Adding LAB as an inoculum will ensure that there is sufficient LAB present to initiate fermentation and prevent the growth of undesirable microorganisms.

This study aimed to determine the bacterial community changes associated with silage fermentation in Malaysia using amplicon metagenomics in grain corn. This is important for improving silage production by analysing bacterial community changes during grain corn ensiling, which can help enhance feed preservation and quality, especially given the high spoilage rate in tropical countries. Understanding microbial interactions can enhance the ensiling process, reduce spoilage, and support sustainable livestock production.

2. MATERIALS AND METHOD

2.1 *Preparation of Grain Corn Silage*

Fresh grain corn and grain corn silage were sampled at Padang Terap, Kedah (6.263313766240991, 100.58890571252691) with pre-approved permission from the private farm owner. A high-speed chopper was used to harvest and chop the whole grain corn plant (leaves, tassel, stalk, and corn fruit). The plant matter was thoroughly mixed before being compressed into a silo. The crop was ensiled for 21 days and the sample was collected from the middle area of the laboratory silo weighed at 400 g and stored at - 20°C freezer for subsequent analysis.

2.2 *Physico-Chemical Analysis of Naturally Fermented Grain Corn Silage*

The water-soluble carbohydrates (WC), dry matter (DM), organic acid content, crude protein (P), and pH values of the fresh grain corn and grain corn silage were measured. Using the 934.01 AOAC method (2016) [16], the dry weight of fresh fodder crop and silage was measured and calculated by drying the sample at 115°C in an Etuve AC120 oven (Froilabo, France) until the DM content was consistent. The water extracts were used in determining the pH value, WC and organic acid content. Whereas the Kjeldahl method was used to determine P according to the AOAC method (2016) [16]. The sample stored at -20°C was homogenising with distilled water at a 10× dilution, then, filtering it through four layers of cheesecloth, and centrifuged water extracts for 20 minutes at 20,000 × g and 4°C. A pH tabletop meter (STARA2110; ThermoScientific, England) was used to measure the pH of fresh crops and silage. For additional analysis, the leftover water extract was stored at -20°C. WC was calculated using the procedures outlined by Dubois et al. (1956) [17]. The concentrations of lactic acid, acetic acid, butyric acid, and propionic acid were determined by using G1321A HPLC manufactured by Agilent Technologies (Germany).

2.3 *Bacterial Enumeration*

Fresh crops and silage were used to count the microbial population. An aqueous extract of the samples was prepared by homogenise 10x dilution using a blender (MX-GM1011; Panasonic, Malaysia) at a 60-second interval at 10 x dilution with 10 g of the sample and 90 mL of sterile saline (0.85%) solution. Then, it was filtered using a sterile Whatman filter paper (No. 40) and underwent serial dilution. MRS (CM361; Oxoid Ltd.) and nutritional agar (NA, CM1160; Oxoid Ltd.) were the culture media used. MRS plates were incubated at 30°C for 48 hours (anaerobically) and NA plates (aerobically) for 24 hours. Aerobic and LAB bacterial counts were measured in CFU/g FM−1 as viable microorganism counts.

2.4 *16S rRNA Gene Amplicon Sequencing*

A mixture of 10 grams of ensiled crops and 50 mL of sterile saline (0.85%) was made. Following the manufacturer's instructions, the sample was filtered, and extracted using the Qiagen DNeasy Blood & Tissue Kit. Nanodrop (ND-2000; Thermo Scientific, USA) was used to determine the concentration of DNA. Paired of primers used was 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) with sample-specific barcodes, the 16S rRNA gene (V3–V4) was amplified. Apical Scientific Sdn Bhd handled the pooling, sequencing, and library preparation. Following the Illumina-recommended technique for pooling and normalisation, the libraries were prepared for next-generation sequencing on the MiSeq platform 300 PE.

2.5 Sequence data analysis

Using BBDuk from the BBTools package (https://sourceforge.net/projects/bbmap/), sequence adaptors and low-quality reads were first removed from paired-end reads. With USEARCH v11.0.667 (https://www.drive5.com/usearch/), the forward and reverse readings were combined. Sequences sequenced on the MiSeq platform that were greater than 600 bp or shorter than 150 bp were excluded from further processing. After that, reads were aligned using 16S rRNA (SILVA Release 132) and examined using VSEARCH v2.6.2 for chimeric errors. Following quality evaluation, UPARSE v11.0.667 was used to cluster reads de novo into OTUs at 97% similarity. Rare OTUs with fewer than two reads (doubleton), which are frequently false, were eliminated from further analysis. Using QIIME V1.9.1 against the Silva 16S rRNA database, the taxonomic assignment of OTU was accomplished (version 132). The National Center for Biotechnology Sequence Read Archive received the sequence data as fastq files, which were then uploaded under the Bio Project accession number PRJNA497711.

2.6 Statistical analyses

The log-transformed microbial enumeration data were used before statistical analysis. To investigate the variations between samples, statistical analyses were carried out using the GLM procedure of the Statistical Package for Social Sciences (SPSS Version 19.0, USA). For varying sample means, Gabriel's honest significant difference test was employed, and significance was established at $p < 0.05$. The MicrobiomeAnalyst web platform, which can be accessed at https://www.microbiomeanalyst.ca/45, was utilised to do bioinformatics analysis. Files containing taxa and metadata for the OTU table were uploaded. The minimum count of four, the 20% prevalence in samples from the low-count filter, and the 10% sample removal from the low-variance filter were the criteria used to filter the data. Total sum scaling was used to scale the data; neither data transformation nor data rarefying was done. Significant differences between groups were determined using the ANOVA/t-test with a p-value of less than 0.05 for alpha diversity. Based on the Bray–Curtis distance, beta diversity was computed, and PERMANOVA was used to compare the groups statistically. A feature-level analysis using an LDA score > 4.0 capped at the initial p-value < 0.05 was carried out using LEfSe. Co-occurrence network analysis was used to assess how bacteria interact in both silage and fresh fodder. SparCC analysis was utilised to calculate the correlations, yielding correlation values greater than 0.5 and 200 permutations with p-values less than 0.05000). Based on each crop, a CCA analysis of noteworthy physicochemical data and differentially abundant taxa was built.

3. RESULTS AND DISCUSSION

3.1 Silage Fermentation Characteristics Imply Ensiling Occurred After 21 Days.

After 21 days of ensiling, water-soluble carbohydrates (WC) in grain corn decreased significantly ($p < 0.05$), while pH increased to 3.80. Crude protein content increased, whereas lactic acid bacteria (LAB) colony-forming units (CFU) declined. Acetic acid was recorded at 2.47% DM, with no detectable propionic or butyric acid. The Lactic Acid/Acetic Acid ratio in G-D21 was 1.05.

Table 1. Fermentation analysis of fresh grain corn and corn silage after ensiling for 21 days.

Sample	G-D0	G-D21
Dry Matter (%)	23.05 ± 1.937^b	27.49 ± 2.546^b
pH	3.55 ± 0.015^a	3.80 ± 0.061^b
Water Soluble Carbohydrates (%DM)	1802.02 ± 726.648^a	1485.89 ± 343.409^a
Lactic Acid (%DM)	ND	2.52 ± 0.615
Acetic Acid (%DM)	ND	2.47 ± 0.302
Butyric Acid (%DM)	ND	Nd
Propionic Acid (%DM)	ND	Nd
Crude Protein (% DM)	23.01 ± 1.853^a	25.84 ± 3.373^a
Viable aerobic bacteria (Log CFU/FM)	7.38 ± 1.930^a	7.66 ± 1.636^a
Viable LAB (Log CFU/FM)	6.78 ± 1.179^a	3.04 ± 0.072^a
Lactic Acid /Acetic Acid ratio	ND	1.05 ± 0.371

G-D0 fresh grain corn, G-D21 grain corn silage, nd not determined, ND not detected, FM fresh materials. Values in the same row followed by different letters are significantly different with *p*-value<0.05.

The ideal DM content for making a good silage quality is usually between 30% and 40% DM [19] but the DM values of fresh grain corn and grain corn silage in this study were less than the optimal value. This might make the silage prone to spoilage because according to Wróbel et al. (2023) [19], fresh crops with less-than-optimal DM can undergo a fermentation driven by Clostridia and it contributes to a potential deterioration. Clostridia fermentation causes an immature production of silage and thus, unable to stop the fungal growth and eventually leads to aerobic spoilage [20]. It can be detected through the concentration of butyric acid (BA) and propionic acid (PA) in the silage, with more than 0.5% DM for each BA and PA [6, 11]. However, in this study, Clostridia spoilage was not observed in grain corn silage as neither BA nor PA was found in the silages (Table 1). The decrease in WC and an increase in pH suggest a shift in fermentation dynamics, likely due to high acetic acid production, which has a weaker pH-lowering effect than lactic acid. These results were interesting as in most research in silage, the pH decreased after ensiling. The equal content valued at 1.05 lactic acid (LA) and AA ratio indicates that a significant portion of fermentation products were a weaker acid which was AA. AA has less significant effectiveness in pH reduction, and it may cause a pH increase. Dominance of *Weissella* and *Lactobacillus* after ensiling indicates slowed fermentation, reduced LA production, and possible proteolysis, leading to pH stabilization.

The physicochemical characteristics of the silage in this study show that silage fermentation was successful. The sensory assessment of silage, such as its odour and colour, can also offer a supplementary review in addition to its physicochemical characteristics.

3.2 Bacterial communities shifted to homofermentative and heterofermentative LAB.

16S rRNA sequencing (V3–V4) of six samples yielded 310,145 reads grouped into 408 operational taxonomic units (OTUs) at 97% similarity. In the rarefaction curve, a plateau was attained for every sample. Similar rarefaction curves in the duplicate sample were classified based on the day of ensiling; G-D21 displayed a higher species richness than G-D0 (Figure 1).

Table 2 displays the percentage abundance of bacterial genera. *Pseudomonas* (41.81%) and *Leuconostoc* (45.59%) were the two main bacterial genera in fresh grain corn (Figure 2, Table 2). But upon ensiling, these genera became less common (Figure 2). *Weissella* was only present in fresh materials at 8.34%, while *Lactobacillus sp*. was only present at 0.14%. After ensiling, homofermentative and heterofermentative *Lactobacillus sp*. increased remarkably by 31.16% and *Weissella* up to 38.98%.

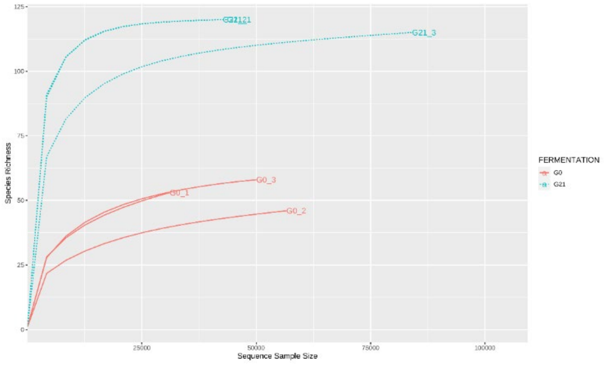


Fig. 1. Rarefaction curve of observed operational taxonomic units (OTUs) for fresh grain corn and grain corn silage using unrarefied sequences (G-D0, Fresh grain corn; G-D21, Grain corn silage).

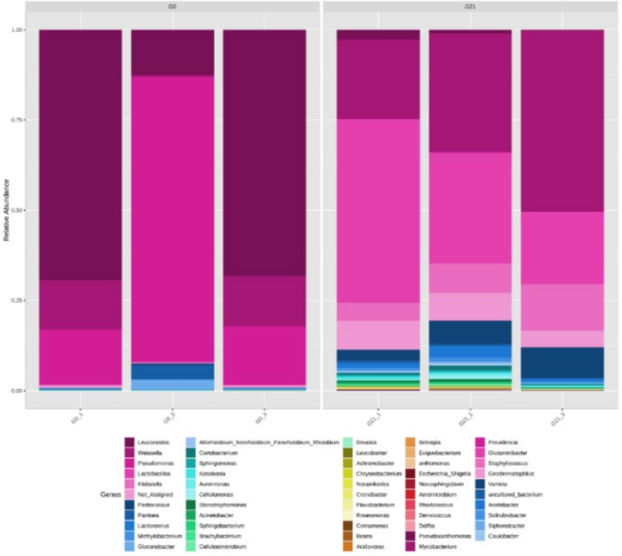


Fig 2. Composition of bacterial communities at the genus level using unrarefied sequences of fresh grain corn and grain corn silage (G-D0, Fresh grain corn; G-D21, Grain corn silage).

Table 2. Percentage abundance of bacterial communities at the genus level.

Genus	G-D0	G-D21
Leuconostoc	45.59	1.12
Pseudomonas	41.81	0.07
Weissella	8.34	38.98
Pantoea	1.61	0.44
Gluconobacter	1.11	0.03
Not_Assigned	0.55	6.02
Methylobacterium	0.37	0.72
Pediococcus	0.19	6.63
Lactobacillus	0.14	31.16
Sphingomonas	0.08	0.39
Aureimonas	0.08	0.30
Allorhizobium Neorhizobium Pararhizobium Rhizobium	0.06	0.50
Curtobacterium	0.02	0.53
Acinetobacter	0.01	0.26
Klebsiella	0.01	9.79
Sphingobacterium	0.01	0.18
Roseomonas	0.01	0.03
Stenotrophomonas	0.00	0.25
Bosea	0.00	0.04
Devosia	0.00	0.11
Lactococcus	0.00	1.52
Novosphingobium	0.00	0.02
Kosakonia	0.00	0.43
Acidovorax	0.00	0.04
Acetobacter	0.00	0.01
Pseudoxanthomonas	0.00	0.01
Belnapia	0.00	0.02
Cronobacter	0.00	0.06
Chryseobacterium	0.00	0.06
Xanthomonas	0.00	0.02
Escherichia_Shigella	0.00	0.02
Acidisoma	0.00	0.00
Rothia	0.00	0.00
Kocuria	0.00	0.00
Herbaspirillum	0.00	0.00
1174_901_12	0.00	0.00
Siphonobacter	0.00	0.01
Burkholderia Caballeronia Paraburkholderia	0.00	0.01
Brachybacterium	0.00	0.14
Leucobacter	0.00	0.07

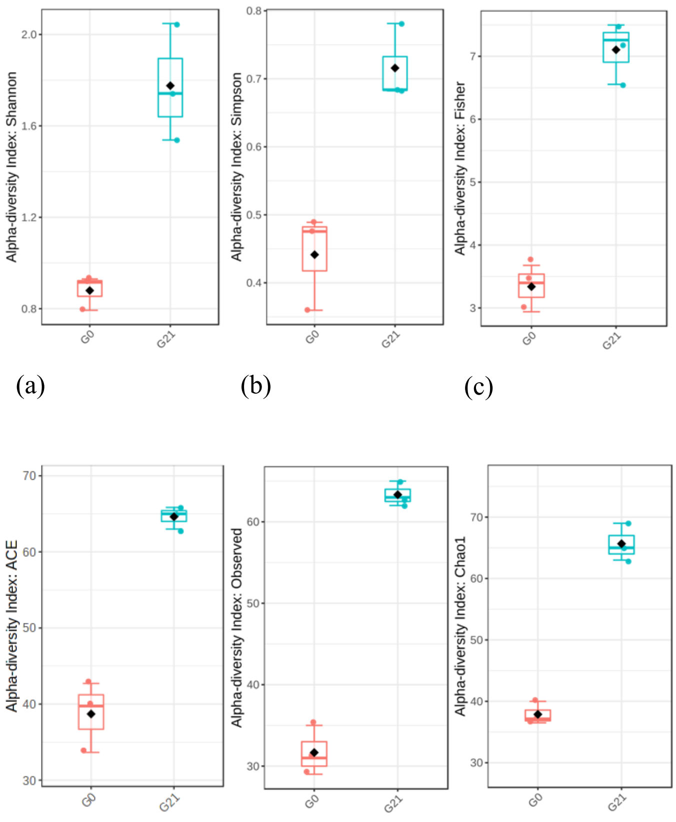
The rarefaction curve indicated that all samples reached a plateau, suggesting that the sequencing data generated was adequate and accurately reflected the diversity of the bacterial community in each sample. The pre-ensiling phase is frequently dominated by facultative aerobes and aerobic bacteria. *Pseudomonas*, an aerobic bacteria was displaced under an anaerobic environment established during ensiling, allowing lactic acid bacteria (LAB) to dominate. The primary bacterial genera in fresh grain corn, including *Pseudomonas, Weissella, Leuconostoc*, and *Pantoea*, have also been reported in other fresh crops, such as soybean [21] and *Moringa oleifera* leaves [22].

After ensiling, *Leuconostoc*, a heterofermentative LAB was displaced with more acid-tolerant LAB such as *Weissella* and *Lactobacillus* were observed in this study. The content of AA and LA was nearly equal which was produced by heterofermentative LAB such as *Weissella* which explained the dominance after ensiling. G-D21 has a smaller percentage of the *Lactobacillus* genus at 31%. Numerous studies claimed that the presence of Lactobacillus was a sign of high-quality silage [22 - 26]. *Leuconostoc* and *Pseudomonas* are commonly found in various environments due to their metabolic capacity to adapt to different conditions [27, 28] *Leuconostoc* is commonly found on plants especially leafy vegetables and sugar cane plants [30].

To evaluate the diversity of bacterial communities, alpha diversity was plotted across samples and visualised for each group sample at the feature level (Figure 3). Following a fermentation period of 21 days, the grain corn's ACE observed OTU, Chao1, Shannon, and Fisher indices increased (p-values = 0.0050, 0.0006, 0.0004, 0.0129, and 0.0007, respectively). The variety and species richness of G-D0 were greater than those of G-D21. Using the Bray–Curtis distance matrix, two-dimensional principal coordinate analysis (PCoA) was shown in Figure 4 where a significant microbial shift was observed between fresh grain corn and grain corn silage (F-value: 5.8879, R-squared: 0.59547, p-value < 0.01).

A linear discriminant analysis (LDA) score > 4.0, capped at p-value < 0.05, was used to perform a linear discriminant analysis effect size (LEfSe) study at the genus level. There were two genera in G-D0 and fifteen differentially abundant genera in G-D21. *Pseudomonas* and *Leuconostoc* were the frequent biomarker taxa of fresh G-D0, according to LEfSE (Fig. 5). *Lactobacillus, Weissella, Klebsiella, Pediococcus, Lactococcus, Curtobacterium, Kosakonia, Cellilomonas, Sphigomonas, Stenotrophomnas, Acinetobacter*, and *Sphongobacterium* were the discriminatory genera for G-D21 (Figure 5).

The increase in alpha diversity indices after ensiling indicates that the microbial community in the silage became richer and more evenly distributed. The separation between fresh forage and silage indicates that the bacterial communities were greatly different after ensiling. According to Sun et al. (2021) [29], bacterial communities in fresh crops showed more diversity than in ensiled crops which can also be observed in corn silage [13, 14] and Napier silage [30, 31]. This suggests a dynamic fermentation process with a diverse microbial ecosystem developing over time. The species richness in grain corn increased after ensiling, and the rarefaction curve result supported the species richness. The increase in evenness-related indices (Shannon, Simpson) suggests that the microbial population became more balanced, with different species present in more comparable abundances rather than being dominated by a few. This result was supported by the observation of the taxonomic composition.



(a) (b) (c)

(d) (e) (f)

Fig. 3. Group samples for alpha diversity indices of bacterial communities before and after ensiling: (a) ACE (F-value: -13.75; p-value = 0.0050**), (b) Observed OTU (F-value: -17.11; p-value = 0.0006***), (c) Chao1 (F-value: -20.49; p-value = 0.0004***), (d) Shannon (F-value: -4.30; p-value = 0.0129*), (e) Simpson (F-value: -3.19; p-value = 0.0539), (f) Fisher (F-value: : -10.54; p-value = 0.0007***), measured at feature level.

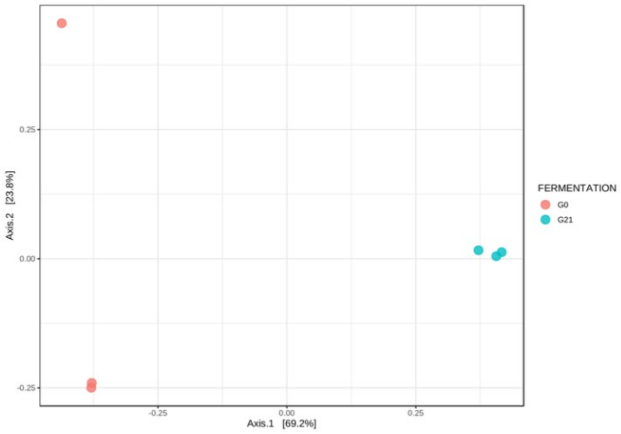


Fig. 4. Principal coordinate analysis (PCoA) using the Bray–Curtis distance between fresh grain corn and grain corn silage. PERMANOVA value, F-value: 5.8879, R-squared: 0.59547, p-value < 0.01.

In silage, aerobic microorganisms remain active for a brief period until anaerobic conditions are established where facultative anaerobic bacteria gradually lower the pH of the silage, leading to the growth of LAB. These LABs typically become the dominant bacteria in the microbiome of the silage at the end of the ensiling process [32]. An outlier in the beta diversity analysis for the G-D0 sample indicates that one of the samples in that group has a microbial community composition that is significantly different from the others can be supported by taxonomic composition. Presumably, during sampling an initial ensiling occurred in one of the G0 samples.

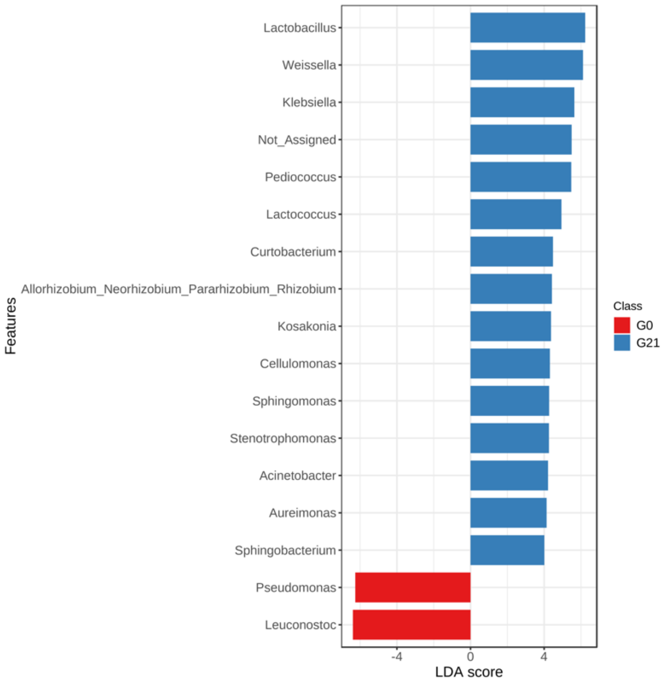


Fig. 5. LEfSe analysis using an LDA effect size between G-D0 and G-D21 at the genus level. The LDA score for the discriminative features threshold on the logarithmic was set to 4.0 at p-value < 0.05 at the original p-value.

Pseudomonas is observed as a biomarker for G-D0 because, before ensiling, the grain corn is exposed to air, allowing aerobic bacteria like *Pseudomonas* to colonise the surface. At Day 0, *Leuconostoc* may be present due to its ability to rapidly initiate fermentation under mildly anaerobic conditions. After 21 days, *Lactobacillus* becomes a biomarker because it dominates the microbial community, driving the primary fermentation process that ensures silage stability. Its abundance at this stage reflects successful fermentation. *Weissella's* presence as a biomarker at G21 suggests it plays a significant role in maintaining the fermentation process. The genus may contribute to a balanced production of lactic and acetic acids, supporting both preservation and spoilage resistance. The taxonomic composition shift from *Pseudomonas* and *Leuconostoc* to *Lactobacillus* and *Weissella* reflects the natural progression of the fermentation process.

To assess the bacterial interactions in fresh forage grain corn and silage, co-occurrence network studies were performed. Using SparCC analysis, the correlations were computed with 200 permutations at a p-value of less than 0.05 and a correlation value of > 0.6 (Figure 6). Table 3 displays the SPARCC correlation for the chosen species. Grain corn's taxonomic network (Figure 6) is made up of 13 nodes with 12 positive and 5 negative relationships. There was a substantial positive association (0.9778) between *Leuconostoc lactis* and *Leuconostoc mesenteroides*, which were prominent in G-D0 before ensiling. *Sphingobacterium multivorum* exhibited a substantial negative connection (r = −1) with *Leuconostoc lactis* and *Lactococcus garvieae* while having a strong positive correlation with *Acinetobacter calcoaceticus* (r = 1).

Table 3 Taxa-taxa correlation in grain corn using SPARCC set at permutation value of 200, *p*-value < 0.05, correlation threshold of > 0.3.

Taxa 1	**Taxa 2**	**Correlation**	***p*-value**
Lactobacillus fermentum	*Lactobacillus brevis*	0.99	0.020
Lactobacillus sp	*Lactobacillus casei*	0.95	0.005
Agrobacterium vitis pv musae	*Acinetobacter calcoaceticus*	0.94	0.020
Xanthomonas sacchari	*Chryseobacterium indologenes*	0.92	0.010
Leuconostoc lactis	*Lactococcus garvieae subsp garvieae*	0.91	0.045
Pseudomonas oryzihabitans	*Acinetobacter calcoaceticus*	0.91	0.020
Pseudomonas oryzihabitans	*Lactococcus garvieae subsp garvieae*	0.90	0.015
Xanthomonas sacchari	*Lactococcus garvieae subsp garvieae*	0.90	0.020
Xanthomonas sacchari	*Pseudomonas oryzihabitans*	0.88	0.020
Lactobacillus sp	*Lactobacillus fermentum*	0.87	0.030
Chryseobacterium indologenes	*Agrobacterium vitis pv musae*	0.86	0.045
Lactobacillus sp	*Lactobacillus brevis*	0.85	0.030
Pseudomonas oryzihabitans	*Agrobacterium vitis pv musae*	0.85	0.035
Lactococcus garvieae subsp garvieae	*Acinetobacter calcoaceticus*	0.83	0.020
Xanthomonas sacchari	*Agrobacterium vitis pv musae*	0.79	0.045
Lactococcus garvieae subsp garvieae	*Chryseobacterium indologenes*	0.74	0.030
Lactococcus garvieae subsp garvieae	*Lactobacillus sp*	-0.67	0.050
Pseudomonas oryzihabitans	*Lactobacillus sp*	-0.71	0.030
Lactococcus garvieae subsp garvieae	*Lactobacillus casei*	-0.71	0.045
Xanthomonas sacchari	*Lactobacillus fermentum*	-0.74	0.035
Xanthomonas sacchari	*Lactobacillus brevis*	-0.76	0.015
Lactococcus garvieae subsp garvieae	*Lactobacillus fermentum*	-0.81	0.020
Lactococcus garvieae subsp garvieae	*Lactobacillus brevis*	-0.82	0.030
Pseudomonas oryzihabitans	*Lactobacillus fermentum*	-0.85	0.020
Pseudomonas oryzihabitans	*Lactobacillus brevis*	-0.86	0.015

Leuconostoc lactis	*Acinetobacter calcoaceticus*	-1.00	0.045

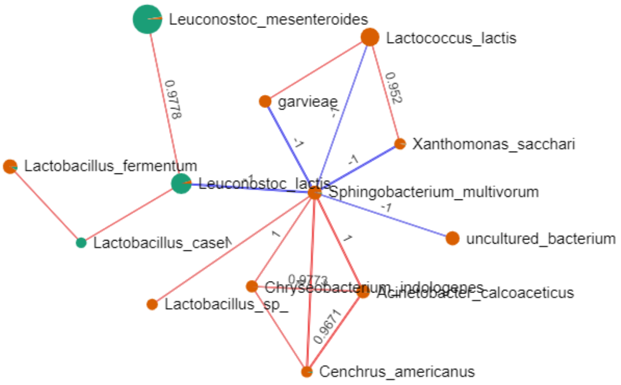


Fig. 6. Taxa-taxa interaction using SPARCC set at permutation value of 200, p-value < 0.05, correlation > 0.3 at species level between G-D0 and G-D21. Orange edges represent a positive correlation while blue edges represent a negative correlation.

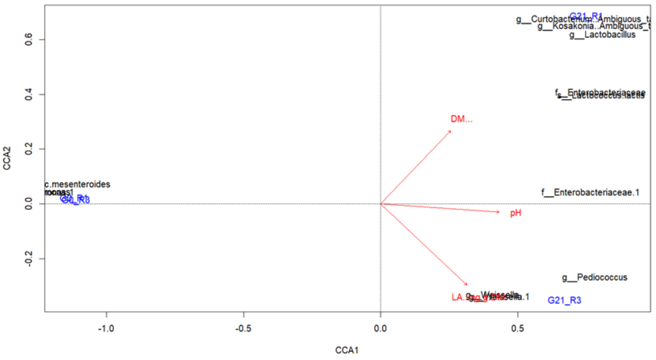
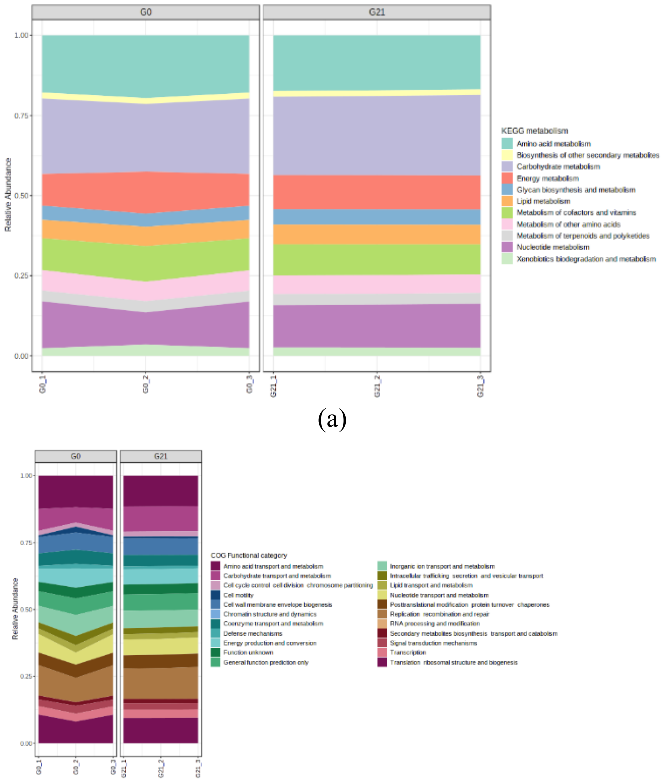


Fig. 7. CCA analysis of correlation among physicochemical properties and bacterial taxa of G-D0 and G-D21 (total variance explained: 92.5%, p > 0.05).

The co-occurrence network analysis examines the relationships between the different bacterial species in the silage. The strong positive correlations between heterofermentative LABs, such as *Leuconostoc lactis* and *Leuconostoc mesenteroids*, suggest their cooperative relationship in colonising fresh crops. These species are common in raw dairy cows' milk, suggesting the possibility of horizontal transfer from forage to milk, although the probability is low [32].

Elevated levels of LA in G-D21 are associated with the abundance of *Lactobacillus* and *Paediococcus*. These bacteria produce LA as a principal product of silage fermentation [33]. *Weissella* and *Paediococcus* are well-suited to the conditions present in silage after 21 days of ensiling. Their prevalence is supported by the moderate pH and high Lactic Acid/Acetic Acid ratio observed. The presence of *Paediococcus* at after ensilling helps stabilise the silage by maintaining a low pH through continuous LA production, which helps inhibit undesirable microbial growth. As the anaerobic condition has been created, the LAB grows rapidly and successfully ferments the available water-soluble carbohydrates left in the aerobic phase into LA, AA, ethanol, and carbon dioxide [36], explaining the correlation of physicochemical analysis and biomarker taxa as in the CCA analysis.

Specifically in a tropical region where silage is easily spoilt and produced at low quality, the prediction of metabolic activities during crop ensiling may offer a thorough insight into the process to ensure that it can produce high-quality silage. Comparing the OTUs with the KEGG database using Tax4Fun, the bacterial community OTUs were utilised to forecast the functional profiles of the Kyoto Encyclopedia of Genes and Genomes (KEGG).



(a)

(b)

Fig. 8. Stacked bar chart of relative abundance of (a) KEGG metabolism and (b) COG functional categories across samples.

The beta diversity observed in Figure 9 indicates that the predicted functional profiles for the KEGG pathway between fresh and corn silage were distinctly separated. Through LEfSe analysis, eight COG Functional Categories were observed in Figure 10 with the LDA score set at 3.0 and FDR-adjusted at p-value < 0.05. The distribution between replicates in G-D0 was observed. After ensiling, carbohydrate transport and metabolism, transport, and catabolism, post-translational modification protein turnover chaperones, secondary metabolite biosynthesis, cell cycle control, and cell division and chromosome partitioning were supplemented.

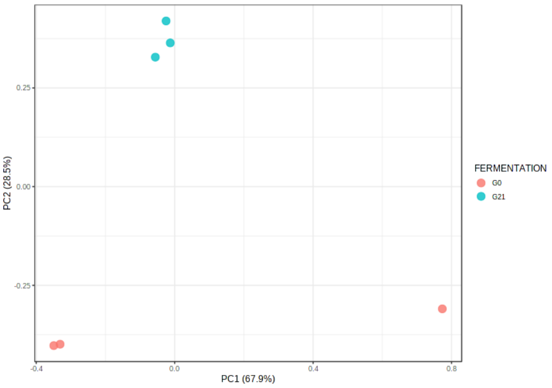


Fig. 9. PCoA between fresh crops and silage on KEGG functional profiles determined using the Euclidean distance algorithm predicted by Tax4Fun.

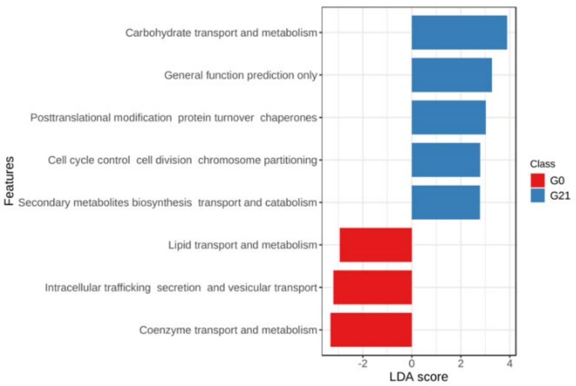


Fig. 10. LEfSe analysis using LDA set to 3.0 at FDR-adjusted *p*-value < 0.05 of the class of COGG functional category using Tax4Fun.

Carbon metabolism and secondary metabolites biosynthesis transport and catabolism differential abundance in grain corn silage which indicates that there is active utilisation of the available substrate to produce organic acid during ensiling. The abundance of fermentative bacteria after ensiling which generate various metabolites can be observed through the COGG function increased after ensiling and the clear separation of KEGG functional profiles in Figure 9. There is further discussion through the data observed on the functions of bacterial communities involved in silage fermentation from the functional prediction, however, the prediction is hypothetical as it used a 16S rRNA gene. It can be validated through other omics studies such as transcriptomics for clear indications.

4. CONCLUSION

After ensiling, the bacterial communities changed significantly, becoming dominated by *Weissella, Pantoea, Pseudomonas, Leuconostoc,* and *Lactobacillus*. Ensiling tropical forages in Malaysia is challenging due to the high temperature and humidity. However, physicochemical analyses indicated that the silage produced was of good quality, with successful LAB dominance following heterofermentative ensiling. The interactions between different taxa revealed positive cooperation among heterofermentative LAB species, along with a negative correlation between LAB and plant pathogens. LAB showed a high abundance in grain corn silage, with a significant amount of LA detected. The main limitation of this study is its focus on grain corn, a single type of tropical forage, which may not reflect the bacterial community dynamics in other forage types. Future research should investigate the ensiling process and bacterial communities across a wider variety of forage crops at different geographical locations to enhance the applicability of the findings. In addition, exploring the use of different silage additives and their effects on microbial community structure and silage quality could further optimize the ensiling process.

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