Doctoral Thesis

Bacterial and fungal microbiota involved in the anaerobic storage of forages

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CONTENTS

PREFACE

The experiments described in this dissertation were carried out at the Graduate school of Environmental and Life Science (Doctor's course), Okayama University, Japan, from April 2019 to March 2022, under the supervision of Professor Naoki NISHINO.

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for the degree, diploma, or other professional qualifications.

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Tables and Figures

CHAPTER 1 General Introduction

Microbiota analysis using DNA information has become more sophisticated and easily accessible in the past 20 years (McAllister et al., 2018). Many studies have been conducted to elucidate the microbiota involved in fermentation and aerobic deterioration of silage, and attempts to develop a novel microbial inoculant are still in progress. With the improvement of harvesting and packaging machinery, techniques for controlling silage quality have been steadily updated. Although methods for microbiota analysis have progressed, most studies have confirmed the well-established knowledge that the rapid growth of lactic acid bacteria (LAB) after sealing determines the success or failure of silage fermentation. Even now, the silo-to-silo and year-to-year quality variations remain unsolved, and mold growth during long-term storage of bale silage is still unavoidable. Likewise, *Lactobacillus buchneri* and its related hetero-fermentative LAB species have been the only choice to prevent aerobic deterioration for more than two decades (R. E. Muck et al., 2018).

The acetic acid fermentation found in tropical grass silages is also a topic for which the mechanisms and control procedures have not been clarified. Because acetic acid fermentation is inevitable even with molasses addition, the lack of soluble sugars is unlikely to be the main factor (Nishino et al., 2012). In addition, acetic acid was not necessarily dominant during the initial stages of fermentation. Lactic acid may be prevalent during the first week of ensiling, but lactic acid can decrease and be entirely or partly replaced with acetic acid (Nishino et al., 2012)(Rodriguez et al., 1989)(Parvin & Nishino, 2009). The fermentation pathway that accounts for this phenomenon remains unclear. The metabolism of lactic acid by *L. buchneri* to produce acetic acid and 1,2-propanediol is one possibility (Oude Elferink et al., 2001), but 1,2-propanediol and its metabolite, 1-propanol, have not been found in large amounts in tropical grass silages. *Acetobacter* spp., that produce acetic acid through oxidizing ethanol, are rarely detected, and their metabolism under aerobic conditions does not explain the decrease in lactic acid levels (Bartowsky & Henschke, 2008).

The fact that most microbiota analyses have focused on bacteria may be why we still lack an understanding of acetic acid fermentation in tropical grass silage. Even if an-aerobic conditions are ensured, a low fungal population can survive ensiling and may activate their metabolism if necessary. Various yeasts, such as *Candida*, *Hansenula*, *Saccharomyces*, and *Torulopsis* spp., can produce lactic acid, acetic acid, and ethanol under anaerobic conditions, although their presence in silage is considered undesirable (McDonald et al., 1991). The fungal microbiota involved in tropical grass ensiling and whether bacterial and fungal microbiota interact during acetic acid fermentation has not been well examined.

Therefore, this study aimed to clarify the bacterial and fungal microbiota associated with guinea grass ensiling during early and long storage periods. Direct-cut (DC) and wilted (WT) silages were prepared to facilitate and restrict acetic acid fermentation. Furthermore, two storage temperatures (25 and 40°C) were studied to determine how fermentation could be affected by high temperatures that mimic tropical and sub-tropical conditions. Phylogenetic gene markers for bacteria and fungi were used to identify the microbial communities.

CHAPTER 2 Microbiota of Guinea Grass Silage Exhibiting Intensive Acetic Acid Fermentation

This study aimed to gain insights into the bacterial and fungal microbiota associated with the acetic acid fermentation of tropical grass silage. Direct-cut (DC, 170 g dry matter [DM]/kg) and wilted (WT, 323 g DM/kg) guinea grass were stored in a laboratory silo at moderate (25℃) and high (40℃) temperatures. Bacterial and fungal microbiota were assessed at 3 days, 1 month, and 2 months after ensiling. Lactic acid was the primary fermentation product during the initial ensiling period, and a high *Lactococcus* abundance (19.7–39.7%) was found in DC silage. After two months, the lactic acid content was reduced to a negligible level, and large amounts of acetic acid, butyric acid, and ethanol were found in DC silage stored at 25℃. The lactic acid reduction and acetic acid increase were suppressed in DC silage stored at 40℃. Increased abundances of *Lactobacillus*, *Clostridium*, and *Wallemia*, and decreased abundance of *Saitozyma*, *Papiliotrema*, and *Sporobolomyces* were observed in DC silages from day 3 to the end of the 2 month period. Wilting suppressed acid production, and lactic and acetic acids were found at similar levels in WT silages, regardless of the temperature and storage period. The abundance of *Lactobacillus* (1.72–8.64%) was lower in WT than in DC silages. The unclassified Enterobacteriaceae was the most prevalent bacteria in DC (38.1–64.9%) and WT (50.9–76.3%) silages, and the abundance was negatively related to the acetic acid content. Network analysis indicated that *Lactobacillus* was involved in enhanced acetic acid fermentation in guinea grass silage.

2.1. Materials and Methods

Silage preparation

The first growth (44 days after seeding) of guinea grass (Panicum maximum cv. Soirukurin; Snow Brand Seed Co., Ltd., Sapporo, Japan) was harvested at the late vegetative stage on 8 August, 2019. The grass was chopped using a forage cutter at a theoretical length of 13 mm immediately after harvest and after field wilting for 4 h. A total of 300 g of pre-ensiled material was packed in a plastic pouch without any additives, and the air was removed using a vacuum sealer. Silos were prepared in triplicate and stored at 25 and 40℃ for 3 days, and for 1 and 2 months.

Chemical components and fermentation products analyses

The dry matter (DM) contents of pre-ensiled and silage samples were determined at 60℃ for 48 h in a forced-air oven. The pH value, as well as the lactic acid, short-chain fatty acid, alcohol, and ammonia nitrogen contents were determined from the water extracts (Tran Thi Minh et al., 2014). The pH was measured using a glass electrode pH meter. The acids and alcohols were determined using an ion-exclusion polymeric high-performance liquid chromatography method with refractive index detection. A portion of the water extract was passed through a 0.20 μm filter, and 10 μL of the filtrate was injected into an IC-Sep COREGEL-87H column (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The mobile phase used was 0.004 M sulfuric acid, and the flow rate was 0.6 mL/min at 60℃. The ammonia nitrogen content was determined using the phenol–hypochlorite reaction (Broderick & Kang, 1980).

MiSeq preparation

Two silages were chosen at random from triplicate silages and subjected to DNA extraction and subsequent MiSeq analyses (Wali & Nishino, 2020). Frozen samples (5 g) were thawed, and 95 mL of sterilized phosphate-buffered saline (pH 7.4) was added. The samples were shaken vigorously for 10 min and microbial pellets were obtained by centrifugation at 8000× *g* for 15 min. DNA extraction was performed using the repeated bead-beating and column method (Yu & Morrison, 2004), followed by DNA purification using a QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan). Bacterial 16S rRNA genes spanning a hypervariable (V4) region were amplified using the F515 (forward: 5-GTGCCAGCMGCCGCGGTAA-3) and R806 (reverse: 5-GGACTACHVGGGTWTCTAAT-3) primers (Caporaso et al., 2011). Fungal rRNA genes spanning the internal transcribed spacer 2 (ITS2) region were amplified using the gITS7 (forward: 5-GTGAATCATCGARTCTTTG-3') and ITS4 (reverse: 5-TCCTCCGCTTATTGATATGC-3) primers (Ihrmark et al., 2012). A two-round polymerase chain reaction (PCR) was performed to reduce any potential bias of the MiSeq adaptor overhang (Berry et al., 2011). For the V4 region of bacterial 16S rRNA genes, the PCR conditions were as follows: initial denaturation at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 30 s, and a final extension step at 72℃ for 5 min. For the ITS2 region of the fungal rRNA genes, the following conditions were employed: 95℃ for 5 min, followed by 40 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s, and a final extension step at 72℃ for 10 min. The PCR products were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and moved to a second round of PCR with adapter-attached primers. Amplification was performed using the following temperature profiles: one cycle at 94℃ for 2 min, 10 cycles of denaturation at 94℃ for 30 s, annealing at 59℃ for 30 s, extension at 72℃ for 30 s, and one cycle at 72℃ for 5 min for the final extension. The PCR products were purified as described above for the first-round products. The purified amplicons were pair-end sequenced (2 × 250) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan). All sequencing data were received as FASTQ files and deposited in the NCBI Sequence Read Archive under BioProject accession PRJNA783142.

Microbial quantification by real-time PCR

Quantitative PCR (qPCR) was carried out in optical-grade 96-well plates on an AriaMx real-time PCR (RT-PCR) system (Agilent Technologies, Ltd., Tokyo, Japan). For total bacteria, the primers were 16S F (forward: 5′-TCCTACGGGAGGCAGCAGT-3′) and 16S R (reverse: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′) (Peng et al., 2018), and the PCR cycling conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 1 min at 60℃, and 1 min at 72℃. For total fungi, the primers were FF390 (forward: 5'-CGATAACGAACGAGACCT-3') and FR1 (reverse: 5'-AICCATTCAATCGGTAIT-3') (Peng et al., 2018), and the PCR cycling conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 30 s at 50℃, and 30 s at 72℃. The rRNA gene copy numbers quantified by real-time PCR were expressed as log10 copies/g silage.

Bioinformatics analysis

Bioinformatics analysis was performed using the QIIME2 program (Bolyen et al., 2019). The raw paired-end FASTQ reads of bacteria and fungi were demultiplexed using the q2-demux plugin. Primer sequences were removed from the bacterial demultiplexed sequence data for quality control. For fungi, demultiplexed sequence data were trimmed with q2-ITSxpress (Rivers et al., 2018) and an unmerged

ITS2 sequence from a fungal amplicon sequencing dataset was extracted for quality control. DADA2 (Callahan et al., 2016) was used to filter, trim, denoise, and merge the data. Chimeric sequences were removed using the consensus method. For phylogenetic diversity analysis, all observed amplicon sequence variants (Callahan et al., 2017), that is, 100% operational taxonomic units (OTUs), were aligned using the MAFFT program plugin via q2-alignment (Katoh et al., 2002) to construct a phylogenetic tree with FastTree2 via q2-phylogeny (Price et al., 2010). Taxonomic classification was assigned using the SILVA database (version 132), specific for the V3-4 region of 16S rRNA genes, and the UNITE fungal ITS database (version 8.2), specific to the ITS2 region of fungal rRNA genes (Nilsson et al., 2019). All taxonomic classifications were implemented using QIIME2 and assigned using the naïve Bayesian algorithm. The alpha diversity (observed OTUs and Faith's phylogenetic diversity) was estimated using q2-diversity at the OTU level.

Statistical analysis

Data for fermentation products, the alpha diversity of bacterial and fungal microbiota, and relative abundances of major bacterial and fungal genera were subjected to a two-way analysis of variance with the storage period and temperature as the main factors. Duncan's multiple range test was used to determine significant differences between the means at a probability of <0.05. These analyses were performed using IBM SPSS Statistics (Version 26.0, IBM Co., Armonk, NY, USA, 2019)

The beta-diversity of bacterial and fungal microbiota was calculated based on the Bray-Curtis distance, using the filtered genus-level taxa and was visualized using principal coordinate analysis (PCoA) with Primer-E software (version 7, Quest Research Ltd., Auckland, New Zealand). Discriminant vectors with a Pearson correlation >0.7 were considered significant. Correlations between fermentation products and the relative abundance of bacterial and fungal genera were calculated using Spearman's rank correlation coefficient. Significant correlations at a probability of <0.05 between the fermentation products and dominant genera were visualized by network analysis using the Cytoscape software (version 3.8.2) (Shannon et al., 2003).

2.2. Results

Fermentation products

The DM contents of DC and WT guinea grass were 170 and 323 g/kg, respectively. Lactic and acetic acids were not detected in the pre-ensiled materials.

A large amount of acetic acid was produced in the DC silage stored at 25℃ for 1 and 2 months (

Table **2.1**). Lactic acid exceeded acetic acid on day three, but lactic acid decreased substantially with prolonged ensiling. After 1 month, acetic acid increased to 25.1 g/kg DM, which was about 2.5 times higher than that of lactic acid. Butyric acid (9.28 g/kg DM) was also found to be comparable to lactic acid (9.44 g/kg DM). After 2 months, acetic acid (49.5 g/kg DM) and butyric acid (21.6 g/kg DM) were twice as high as after 1 month. A considerable amount of ethanol (15.0 g/kg DM) was also produced, but lactic acid (0.34 g/kg DM) was further lowered to a negligible level.

Table 2.1 Dry matter, pH value, and fermentation products of direct-cut guinea grass silage stored at 25 and 40℃ for 3 days, 1 month, and 2 months.

		DM (g/kg)	pH	Lactic Acid (g/kg DM)	Acetic Acid (g/kg DM)	Ethanol (g/kg DM)	L/A	NH_3-N (g/kg DM)	Propionic Acid (g/kg DM)	Butyric Acid (g/kg DM)
25℃	3 days	164	5.51	15.0 a	9.05 b	3.19 b	1.71 a	1.33 b	ND	ND
	1 month	162	5.51	9.44 ab	25.1 b	4.02 b	0.34 b	2.38 ab	0.90	9.28
	2 months	155	5.37	0.34 b	49.5 a	15.0 a	0.01 b	3.95 a	3.47	21.6
	SE	3.41	0.16	3.56	5.16	2.16	0.20	0.54	–	–
40℃	3 days	168 z	5.24	21.3	6.84 z	1.63 y	3.14 x	1.30 y	ND	ND
	1 month	176 y	5.04	24.9	22.3 y	2.25 y	1.13 y	1.99 x	ND	ND
	2 months	188 x	4.95	25.2	27.4 x	3.98 x	0.93 y	2.02 x	ND	ND
	SE	1.16	0.10	2.95	1.14	0.32	0.39	0.08	–	–
Two-way ANOVA										
	T	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01	<0.05	–	–
	P	0.126	0.319	0.253	<0.01	<0.01	<0.01	<0.01	–	–
	T × P	<0.01	0.761	<0.05	<0.05	<0.05	0.568	0.068	–	–

DM, dry matter; L/A, lactic to acetic acid ratio; ND, not detected; SE, Pooled standard error; T, storage temperature; P, storage period. Values in the same column with different following letters (a–b, x–z) are significantly different. Due to the fact that the interaction was observed for several items, a one-way analysis of variance was performed for the storage at 25 and 40℃ separately.

Intensive lactic acid fermentation was observed during the initial ensiling of DC silage stored at 40℃; the ratio of lactic acid to acetic acid was >3.0 on day three. Unlike the DC silage stored at 25℃, lactic acid did not decrease with prolonged storage, and acetic acid became comparable to lactic acid after 1 and 2 months. Ethanol and ammonia nitrogen also increased with prolonged ensiling, but the increasing pattern was different from that of acetic acid. Butyric acid was not found in the DC silage stored at 40℃, regardless of the ensiling period.

Table 2.2 Dry matter, pH value, and fermentation products of wilted guinea grass silage stored at 25 and 40℃ for 3 days, 1 month, and 2 months.

		DM (g/kg)	pH	Lactic Acid (g/kg DM)	Acetic Acid (g/kg DM)	Ethanol (g/kg DM)	L/A	NH_3-N (g/kg DM)	Propionic Acid (g/kg DM)	Butyric Acid (g/kg DM)
25℃	3 days	354	6.12 a	0.96 b	1.08 c	0.77 c	0.92	0.50 b	ND	ND
	1 month	379	6.05 ab	7.06 a	7.43 b	3.07 b	1.01	1.51 a	ND	ND
	2 months	344	5.84 b	9.36 a	11.6 a	6.98 a	0.84	1.27 a	ND	ND
	SE	17.5	0.06	0.98	1.00	0.49	0.20	0.16	–	–
40℃	3 days	376	6.16 x	1.86 z	2.33 y	1.40 y	0.81	0.68 y	ND	ND
	1 month	419	5.77 y	6.89 y	5.48 y	1.58 y	1.30	1.23 x	ND	ND
	2 months	430	5.45 z	13.1 x	10.3 x	5.25 x	1.27	1.44 x	ND	ND
	SE	20.0	0.05	1.45	1.01	0.56	0.22	0.09	–	–
Two-way ANOVA										
	T	<0.01	<0.01	0.164	0.432	0.068	0.251	0.823	–	–
	P	0.217	<0.01	<0.01	<0.01	<0.01	0.404	<0.01	–	–
	T × P	0.254	<0.01	0.296	0.280	0.085	0.424	0.159	–	–

DM, dry matter; L/A, lactic to acetic acid ratio; ND, not detected; SE, Pooled standard error; T, storage temperature; P, storage period. Values in the same column with different following letters (a–c, x–z) are significantly different. Due to the fact that the interaction was observed for several items, a one-way analysis of variance was performed for the storage at 25 and 40℃ separately.

In WT silages stored at 25 and 40℃, the amounts of lactic acid and acetic acid were similar, that is, the ratio of lactic acid to acetic acid was approximately 1.0 throughout the ensiling period (

Table **2.2**). Acid and alcohol production was suppressed in WT silages; lactic acid, acetic acid, and ethanol were <30 g/kg DM, and no butyric acid was found even after 2 months. Ammonia nitrogen levels were considerably lower than the corresponding levels in the DC silages.

Bacterial microbiota

A total of 810,737 filtered 16S rRNA high-quality sequences were obtained to classify 1101 OTUs from 26 samples. The observed OTUs and the Faith PD of pre-ensiled DC and WT materials were 212 and 22.1, and 205 and 24.5, respectively. These two indices were distinctly decreased after ensiling in both DC and WT silages. The observed OTUs and Faith PD were approximately one-third of those observed in the pre-ensiled materials on day three, and did not change significantly after ensiling (Table 2.3).

Table 2.3 α diversity of the bacterial microbiota of direct-cut and wilted guinea grass silage stored at 25℃ and 40℃ for 3 days, 1 month, and 2 months.

		Direct-cut silage		Wilted silage	
		Observed OTUs	Faith PD	Observed OTUs	Faith PD
25℃	3 days	56.0 b	5.82	91.5	9.89
	1 month	81.5 a	7.01	54.0	6.65
	2 months	73.0 ab	6.51	59.5	7.58
	SE	5.42	0.27	8.73	1.00
40℃	3 days	63.5 x	7.33 x	67.5	8.20 x
	1 month	47.5 y	4.94 y	59.0	5.97 y
	2 months	73.5 x	7.26 x	68.0	7.75 xy
	SE	2.63	0.23	4.63	0.43
Two-way ANOVA					
	T	<0.05	<0.05	<0.05	<0.05
	P	<0.05	0.767	0.562	0.290
	T×P	<0.01	<0.01	0.110	0.522

OTUs, operational taxonomic units; PD, phylogenetic diversity; SE, Pooled standard error; T, storage temperature; P, storage period. Values in the same column with different following letters (a–b, x–y) are significantly different. Because the interaction was observed for several items, one-way analysis of variance was performed for the storage at 25℃ and 40℃ separately.

The five most abundant bacterial genera in the pre-ensiled DC crop were *Pseudomonas* (19.0%), *Pantoea* (18.3%), *Acinetobacter* (13.7%), *Sphingomonas* (9.57%), and *Bacillus* (5.30%), and those in WT crop were *Pseudomonas* (32.5%), *Sphingomonas* (9.55%), unclassified Burkholderiaceae (6.86%), *Chryseobacterium* (5.13%), and *Acinetobacter* (4.92%).

Immediately after ensiling, unclassified Enterobacteriaceae became the most abundant taxon in the guinea grass silage (

Table **2.4**; Figure 2.1). The abundance was only 2.22% in pre-ensiled DC crop, but unclassified Enterobacteriaceae increased to 46.1%, which exceeded *Lactococcus* (37.6%) on day three in DC silage stored at 25℃. After 1 month, unclassified Enterobacteriaceae accounted for 39.3%, whereas the abundance of *Lactococcus* decreased to 10.9% and that of *Lactobacillus* increased to 32.0%. After 2 months, the abundance of unclassified Enterobacteriaceae was 32.7%, followed by *Clostridium* (24.3%) and *Lactobacillus* (19.2%). In DC silage stored at 40℃, the abundance of unclassified Enterobacteriaceae was as high as 60%. *Lactococcus* was the second most abundant on day three, and *Lactococcus* decreased, and *Lactobacillus* became the second most abundant after 1 month, similar to DC silage. Meanwhile, the abundance of *Clostridium* was only 0.18%, and *Lactobacillus* was the second most abundant bacterial taxon in DC silage stored at 40.

Table 2.4 Relative abundances (%) of the eight most abundant bacterial genera in direct-cut guinea grass silage stored at 25 and 40℃ for 3 days, 1 month, and 2 months.

		unclassified Enterobacteriaceae	Lactobacillus	Lactococcus	Pediococcus	Enterococcus	Weissella	Clostridium	Bacillus
25℃	3 days	46.1	6.32	37.6 a	0.81	3.24	2.58	ND	0.01
	1 month	39.3	32.0	10.9 b	0.83	4.79	2.79	4.54	0.15
	2 months	32.7	19.2	10.1 b	0.57	6.34	1.31	24.3	0.02
	SE	5.31	6.69	2.10	0.36	0.83	1.29	–	0.03
40℃	3 days	59.1	3.36 y	20.8 x	6.36 x	3.82 z	4.32	ND	ND
	1 month	50.3	25.3 x	13.0 y	1.59 y	6.37 y	1.82	ND	0.48
	2 months	49.0	23.1 x	11.4 y	3.29 xy	7.23 x	1.38	0.18	2.32
	SE	4.19	3.10	0.87	0.86	0.19	1.33	–	–
Two-way ANOVA									
	T	0.118	<0.05	<0.01	<0.05	<0.01	0.342	0.132	<0.01
	P	<0.05	0.672	<0.05	<0.01	0.084	0.801	0.074	<0.01
	T × P	0.858	0.612	<0.01	<0.05	0.712	0.605	0.139	<0.01

SE, Pooled standard error; T, storage temperature; P, storage period. ND, not detected. Values in the same column with different following letters (a–b, x–z) are significantly different. Due to the fact that the interaction was observed for several items, a one-way analysis of variance was performed for the storage at 25 and 40℃ separately.

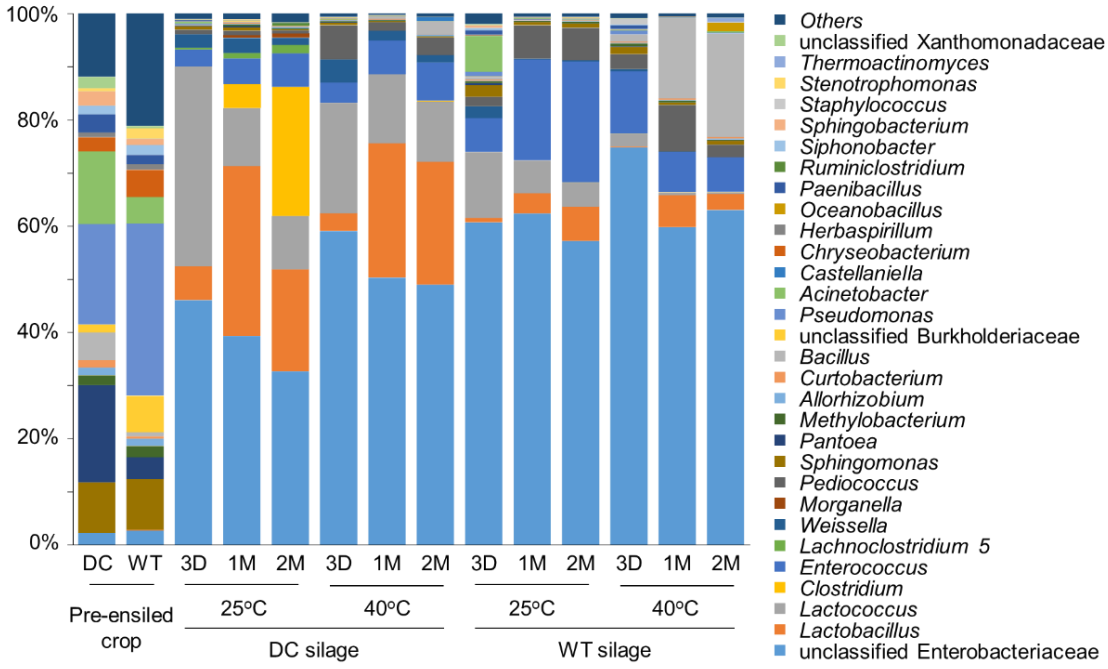

Figure 2.1 Relative abundances of the 30 major genera (>1% in abundance) of the bacterial microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. DC and WT indicate direct-cut and wilted crops and silages. 3D, 1M, and 2M indicate storage for 3 days, 1 month, and 2 months at 25 and 40℃, respectively.

In WT silage, unclassified Enterobacteriaceae appeared more than that in DC silage stored at 25℃; the abundance on day 3 was >60%, followed by that of *Lactococcus* (12.4%) in WT silage stored at 25℃ (

Table **2.5**). The abundance of unclassified Enterobacteriaceae was >70%, and *Enterococcus* (11.8%) was second-most abundant on day three in WT silage stored at 40℃. Unclassified Enterobacteriaceae maintained their abundance at approximately 60% after 1 and 2 months, regardless of storage

temperature. Unlike DC silage, *Enterococcus* (19.0–22.8%) and *Bacillus* (15.1–19.5%) were the second most abundant bacteria after 1 and 2 months of storage at 25 and 40°C, respectively.

Table 2.5 Relative abundances (%) of the eight most abundant bacterial genera in wilted guinea grass silage stored at 25 and 40°C for 3 days, 1 month, and 2 months.

		unclassified Enterobacteriaceae	Lactobacillus	Lactococcus	Pediococcus	Enterococcus	Weissella	Clostridium	Bacillus
25°C	3 days	60.7	0.85 b	12.4 a	1.76	6.43 b	2.25 a	ND	0.52
	1 month	62.4	3.76 ab	6.18 b	6.22	19.0 a	0.19 b	ND	ND
	2 months	57.2	6.47 a	4.52 b	6.12	22.8 a	0.22 b	0.01	0.06
	SE	3.40	1.23	0.75	1.39	1.98	0.09	–	–
40°C	3 days	74.8	0.22	2.35	2.77	11.8	0.44	0.01	1.25
	1 month	59.8	5.99	0.53	8.77	7.61	0.08	0.02	15.1
	2 months	63.0	3.04	0.34	2.39	6.59	0.01	0.00	19.5
	SE	7.65	1.59	0.68	3.06	2.53	0.25	0.00	11.0
Two-way ANOVA									
	T	0.427	<0.05	<0.01	0.165	0.108	<0.01	0.819	0.528
	P	0.277	0.618	<0.01	0.977	<0.01	<0.01	0.286	0.113
	T × P	0.424	0.217	<0.05	0.437	<0.01	<0.01	0.528	0.493

SE, Pooled standard error; T, storage temperature; P, storage period. ND, not detected. Values in the same column with different following letters (a–b) are significantly different. Due to the fact that the interaction was observed for several items, a one-way analysis of variance was performed for the storage at 25 and 40°C separately.

Based on the PCoA plots, pre-ensiled materials were shown to be quite different in the bacterial microbiota from their silages; their microbiota were characterized by *Pseudomonas*, *Pantoea*, *Sphingobacterium*, and others (Figure 2.2). The DC silages opened on day three formed a separate group from those opened after 1 and 2 months. *Lactobacillus* characterized these long-stored DC silages with a high amount of acetic acid. The WT silages formed another group, which was characterized by unclassified Enterobacteriaceae.

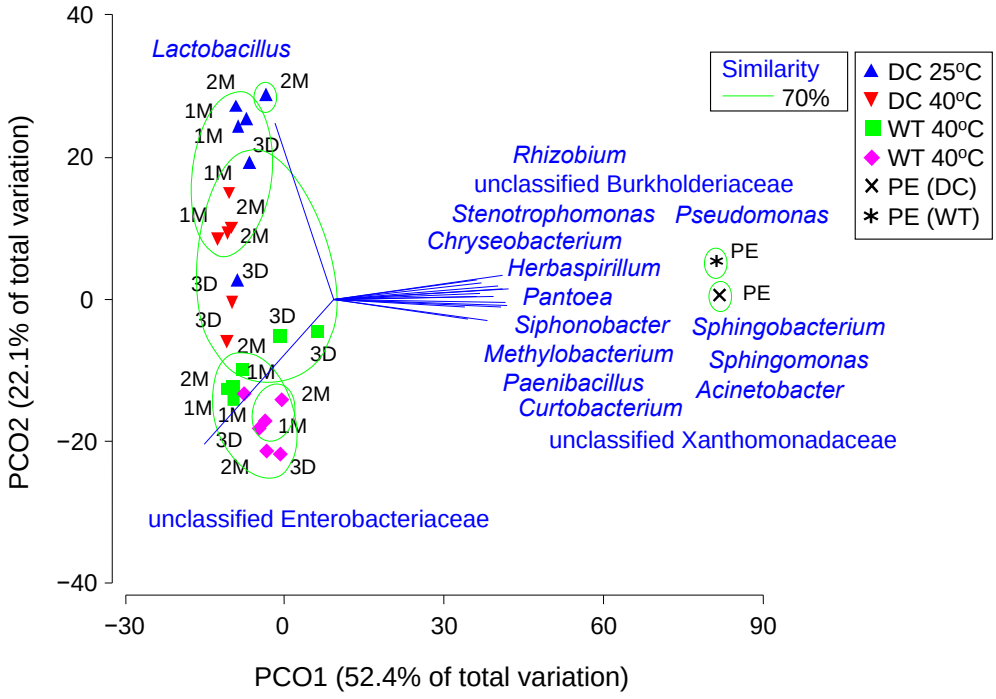

Figure 2.2 Beta diversity of the bacterial microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together on the ordination have a more similar microbiota. The operational taxonomy unit with a Pearson's correlation of >0.7 is overlaid on the plot as vectors. Green circles denote samples enclosed in the same group at a 70% similarity level. PE, DC, WT, 3D, 1M, and 2M indicate pre-ensiled crop, direct-cut silage, wilted silage, silage stored for 3 days, silage stored for 1 month, and silage stored for 2 months, respectively.

Fungal microbiota

A total of 206,864 filtered ITS2 high-quality sequences were obtained to classify 671 OTUs. The observed OTUs and Faith PD of pre-ensiled DC and WT materials were 40 and 12.3, and 60 and 18.8, respectively. On day three, these two indices numerically increased in DC silages and decreased in WT silages. In DC silages, regardless of the storage temperature, the observed OTUs decreased after 1 and 2 months when compared with day three. In WT silage stored at 25℃, the observed OTUs and Faith PD appeared to decrease after 1 and 2 months. These two indices increased after 1 and 2 months in WT silage stored at 40℃ (

Table 2.6 α diversity of the fungal microbiota of direct-cut and wilted guinea grass silage stored at 25℃ and 40℃ for 3 days, 1 month, and 2 months.).

Table 2.6 α diversity of the fungal microbiota of direct-cut and wilted guinea grass silage stored at 25℃ and 40℃ for 3 days, 1 month, and 2 months.

		Direct-cut silage		Wilted silage	
		Observed OTUs	Faith PD	Observed OTUs	Faith PD
25℃	3 days	46.5 a	13.5	45.5	14.6
	1 month	38.0 b	13.4	24.0	10.3
	2 months	34.0 b	14.0	27.0	11.4
	SE	1.55	1.81	7.77	3.13
40℃	3 days	63.0 x	18.3	43.0 z	14.5 z
	1 month	47.5 xy	15.3	70.0 y	19.6 y
	2 months	44.0 y	15.9	83.0 x	24.1 x
	SE	3.62	0.97	2.52	0.35
Two-way ANOVA					
	T	<0.01	0.589	0.234	0.372
	P	<0.01	0.053	<0.01	<0.01
	T×P	0.427	0.555	<0.01	0.065

OTUs, operational taxonomic units; PD, phylogenetic diversity; SE, Pooled standard error; T, storage temperature; P, storage period. Values in the same column with different following letters (a–b, x–z) are significantly different. Because the interaction was observed for one item, one-way analysis of variance was performed for the storage at 25℃ and 40℃ separately.

The five most abundant fungal genera in the pre-ensiled DC crop were *Wallemia* (30.6%), *Saitozyma* (23.4%), *Gibellulopsis* (10.3%), *Sporobolomyces* (7.92%), and *Moesziomyces* (7.34%). In DC silage stored at 25℃, the abundance of *Wallemia* decreased on day three but increased again as ensiling was extended (

Table **2.7**; Figure 2.3). In DC silage stored at 40℃, *Wallemia* was the most abundant throughout the ensiling period, with an abundance of >30% after 2 months, regardless of the storage temperature. The abundances of *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* were higher than those in the pre-ensiled crop on day three, and then decreased to levels lower than those in the pre-ensiled crop.

The five most abundant fungal genera in the pre-ensiled WT crop were *Saitozyma* (15.2%), *Moesziomyces* (14.0%), *Cladosporium* (13.2%), Alternaria (6.39%), and unclassified Dissoconium

(5.35%) (Figure 2.3). Similar to DC silage, a temporal increase in *Saitozyma* on day three was observed in the WT silage (

Table **2.8**). Unlike DC silage, *Wallemia* was not the most abundant, and *Saitozyma*, *Papiliotrema*, and *Sporobolomyces* were the major fungi in WT silages regardless of the storage temperature and ensiling period. A high abundance of *Mucor* (42.7%) was observed in WT silage stored at 25°C after 1 month, because the fungus inhabited one silage at an extremely high abundance. Likewise, *Wallemia* was the most abundant in WT silage stored at 40°C after 2 months, because one silage showed a substantial abundance of *Wallemia*.

Table 2.7 Relative abundances (%) of the eight most abundant fungal genera in direct-cut guinea grass silage stored at 25 and 40°C for 3 days, 1 month, and 2 months.

		Wallemia	***Mucor***	***Saitozyma***	***Hannaella***	***Sporobolomyces***	***Papiliotrema***	***Moesziomyces***	***Cladosporium***
25°C	3 days	6.58 c	0.3	32.7 a	6.90	14.6 a	17.7 a	6.67	1.74
	1 month	47.3 a	ND	4.24 b	1.61	1.97 b	5.42 ab	ND	2.28
	2 months	32.1 b	ND	7.70 b	1.07	1.03 b	0.02 b	1.91	2.45
	SE	3.12	–	4.83	2.54	2.22	2.85	–	0.63
40°C	3 days	22.4	ND	20.8	5.42	8.98	16.1	9.90 x	2.07
	1 month	50.1	ND	12.7	ND	6.44	2.73	0.68 y	9.67
	2 months	42.9	ND	13.0	1.13	2.86	0.40	1.45 y	4.55
	SE	11.6	–	5.92	–	2.16	4.16	1.61	5.05
Two-way ANOVA									
	T	<0.05	–	<0.05	0.087	<0.01	<0.01	<0.01	0.556
	P	0.207	–	0.900	0.593	0.910	0.671	0.323	0.308
	T × P	0.753	–	0.208	0.916	0.134	0.911	0.412	0.618

SE, Pooled standard error; T, storage temperature; P, storage period. ND, not detected. Values in the same column with different following letters (a–c, x–y) are significantly different. Although the interaction was not observed for any items, a one-way analysis of variance was performed for the storage at 25 and 40°C separately.

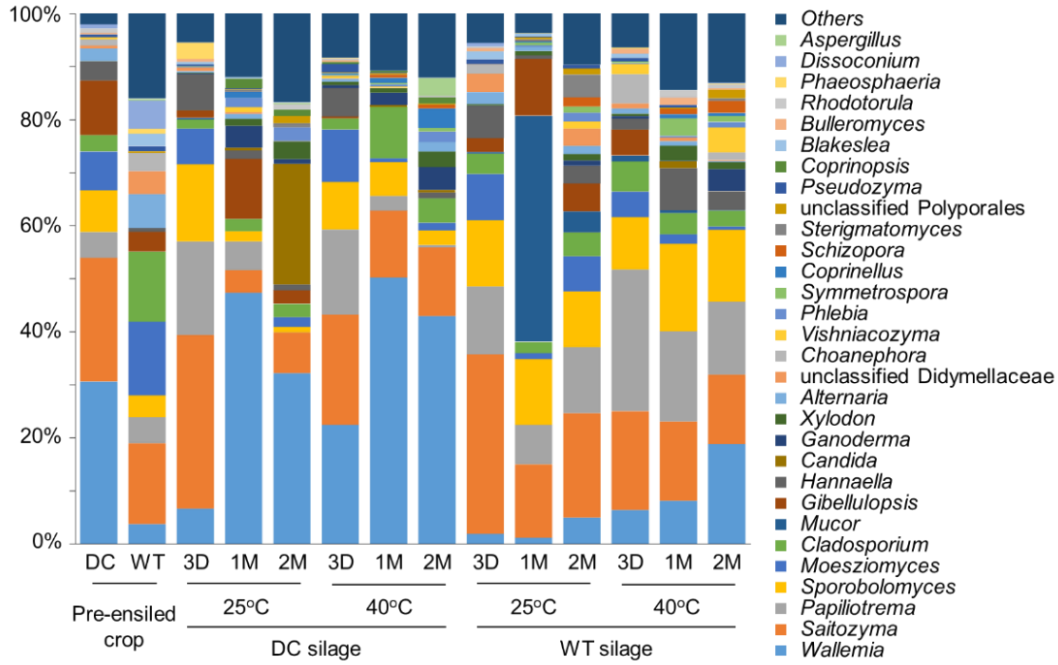

Figure 2.3 Relative abundances of the 30 major genera (>1% in abundance) of the fungal microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. DC and WT indicate

direct-cut and wilted crops and silages. 3D, 1M, and 2M indicate storage for 3 days, 1 month, and 2 months at 25 and 40℃, respectively.

Table 2.8 Relative abundances (%) of the eight most abundant fungal genera in wilted guinea grass silage stored at 25 and 40℃ for 3 days, 1 month, and 2 months.

		Wallemia	Mucor	Saitozyma	Hannaella	Sporobolomyces	Papiliotrema	Moesziomyces	Cladosporium
25℃	3 days	1.82	0.26	33.9 a	6.17	12.5	12.8	8.71 a	3.87
	1 month	1.08	42.7	13.9 b	0.71	12.5	7.42	1.09 b	2.13
	2 months	4.93	3.98	19.7 ab	3.29	10.6	12.4	6.67 a	4.41
	SE	2.92	22.6	3.41	2.50	5.79	3.25	0.86	2.64
40℃	3 days	6.36	1.16	18.6	2.14	9.95	26.7	4.82 x	5.63
	1 month	8.08	0.58	14.9	7.91	16.6	17.0	1.75 y	3.99
	2 months	18.7	0.11	13.1	3.54	13.7	13.7	0.62 y	3.00
	SE	6.78	0.75	3.27	1.85	5.01	8.10	0.52	1.61
Two-way ANOVA									
	T	0.321	0.401	<0.05	0.910	0.826	0.458	<0.01	0.747
	P	0.095	0.294	<0.05	0.548	0.737	0.154	<0.01	0.694
	T × P	0.673	0.397	0.127	0.107	0.806	0.609	<0.01	0.709

SE, Pooled standard error; T, storage temperature; P, storage period. ND, not detected. Values in the same column with different following letters (a–b, x–y) are significantly different. Although the interaction was not observed for any items, a one-way analysis of variance was performed for the storage at 25 and 40℃ separately.

The PCoA plots did not clarify the differences in fungal microbiota between the pre-ensiled crop, DC silage, and WT silage (Figure 2.4). At the 70% similarity level, DC and WT silages opened on day three formed a group, while others emphasized individual silo-to-silo variations. The genera characterized by the silages on day three were *Saitozyma*, *Sporobolomyces*, and *Papiliotrema*. One WT silage, stored at 25℃ for 1 month, was separately characterized by *Mucor*.

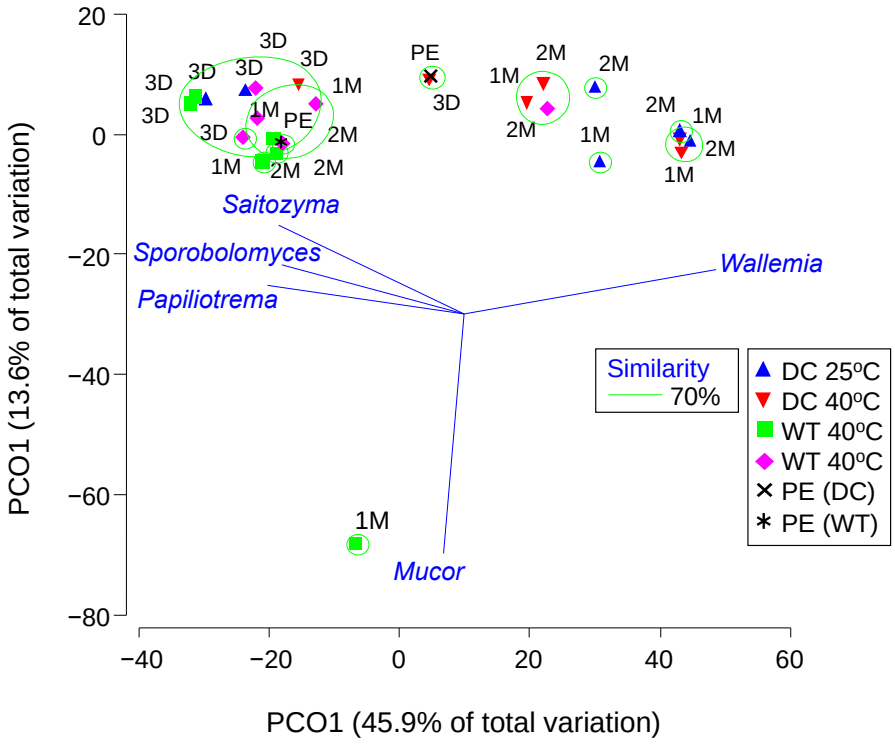

Figure 2.4 Beta diversity of the fungal microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together on the ordination have a more similar microbiota. The operational taxonomy unit with a Pearson's

correlation of >0.7 is overlaid on the plot as vectors. Green circles denote samples enclosed in the same group at a 70% similarity level. PE, DC, WT, 3D, 1M, and 2M indicate pre-ensiled crop, direct-cut silage, wilted silage, silage stored for 3 days, silage stored for 1 month, and silage stored for 2 months, respectively.

Relationships between the fermentation products and core genera of bacteria and fungi

A correlation network indicated that acetic acid was positively related to ammonia, butyric acid, propionic acid, and ethanol (Figure 2.5). A positive relationship between acetic acid and the abundance of *Lactobacillus*, *Clostridium*, *Wallemia*, and *Candida*, and a negative relationship between acetic acid and the abundance of unclassified Enterobacteriaceae, *Pediococcus*, *Saitozyma*, *Sporobolomyces*, *Papilioterma*, *Moesziomyces*, and *Hannaella* was also demonstrated. Lactic acid was negatively related to the fungal genus *Gibellulopsis*, but unrelated to the bacterial genus *Lactobacillus*. *Clostridium* was positively related to acetic acid, propionic acid, butyric acid, and ammonia nitrogen content.

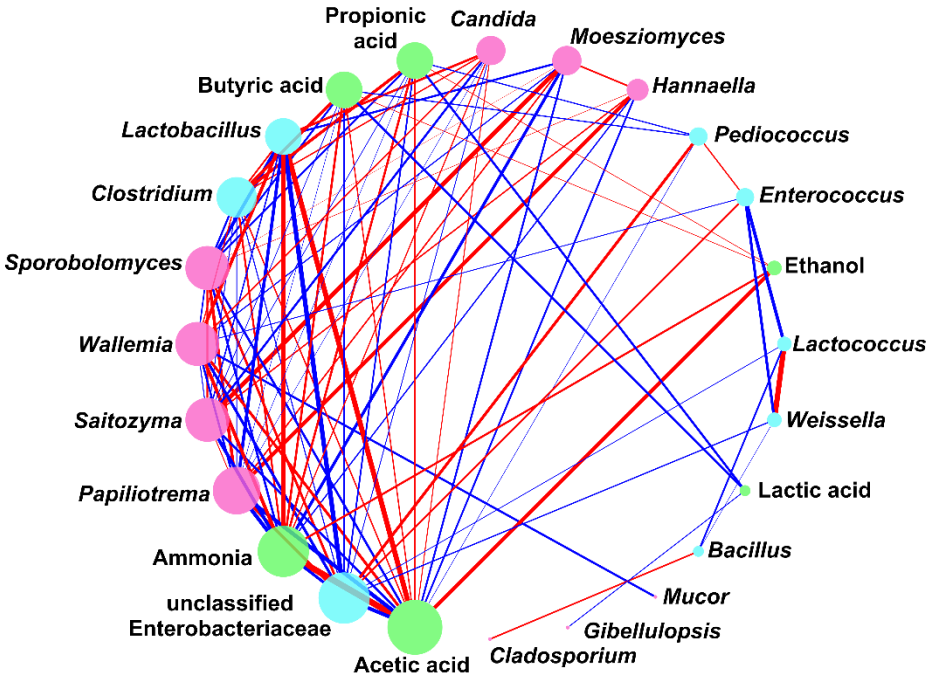

Figure 2.5 Correlation network diagram for fermentation products and major bacterial and fungal genera of direct-cut and wilted guinea grass silage. Green, blue, and purple nodes represent the fermentation product, bacterial genus, and fungal genus, respectively. The node size represents the degree of centrality, which is calculated as the number of edges connecting a node to others. The Spearman's Rho of two nodes is indicated by the edge width, with red and blue edges describing the positive and negative relationships between the two nodes.

2.3. Discussion

Enhanced acetic acid production was observed in both the DC and WT silages. However, according to the lactic-to-acetic acid ratio, a typical intensified acetic acid fermentation was observed in DC silage, especially after day three at both 25℃ and 40℃ storages. In DC silage stored at 25℃, high amounts of butyric acid (21.6 g/kg DM) and ammonia nitrogen (3.95 g/kg DM) were found with a large abundance of *Clostridium* (24.3%) after 2 months. Thus, the increase in acetic acid during prolonged ensiling could be due to the mixed activity of undefined acetic acid-producing bacteria and *Clostridium*. In WT silage, regardless of the storage temperature, the lactic-to-acetic acid ratio was approximately 1.0 throughout

the ensiling period; hence, fermentation should be considered to be driven by hetero-fermentative LAB species.

The PCoA and network analyses showed that *Lactobacillus*, unclassified Enterobacteriaceae, *Wallemia*, *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* could be involved in acetic acid fermentation. *Lactobacillus* and *Wallemia* were identified as promoting factors and unclassified Enterobacteriaceae, *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* as suppressing factors. Regardless, the abundance of *Lactobacillus* was most distinctively related to changes in the acetic acid content during fermentation.

The finding that *Lactobacillus* may be involved in the increase of acetic acid in tropical grass silage is similar to that in our previous study (Nishino et al., 2012; Parvin & Nishino, 2009). *L. plantarum* became detectable with the increase of acetic acid by semi-quantitative denaturing gradient gel electrophoresis analysis; hence, the metabolism of lactic acid by *L. plantarum* to acetic acid under sugar-deficient conditions was considered to be explained (Parvin & Nishino, 2009). Since the MiSeq platform was used, classification at the species level was difficult in this study. Likewise, it is not clear if the metabolism of lactic acid to acetic acid is well shared within the genus *Lactobacillus*. Regardless, the findings of this study confirm that *Lactobacillus* can be involved in intensified acetic acid production in tropical grass silage. The metabolism of lactic acid by *L. buchneri* to acetic acid and 1,2-propanediol could account for the excessive levels of acetic acid (Oude Elferink et al., 2001), but 1,2-propanediol and its potential metabolite, 1-propanol, were not detected in significant amounts. Lactic acid rarely accumulates in the gut and, even if produced, it can be metabolized to acetic, propionic, and butyric acid (Duncan et al., 2004). *Veillonella*, *Selenomona*s, and *Megasphaera* are well-known lactate-utilizing and acetate-producing bacteria (Duncan et al., 2004), but these species have not been found in silage and were not detected in this study.

Interestingly, unclassified Enterobacteriaceae were maintained at 40–50% in DC silage and >60% in WT silage. Li et al. (D. Li et al., 2019) also reported that *Enterobacter* was detectable at >60% after 2 months in the bacterial microbiota of king grass, paspalum, and stylo silage. Accordingly, *Enterobacter* is considered to be a major bacterial factor that accounts for acetic acid fermentation (D. Li et al., 2019). However, they did not examine fermentation products and microbiota during the initial ensiling, such as within 3 days, which was conducted in the present study. Furthermore, although Enterobacteriaceae are known to produce large amounts of ethanol and 2,3-butanediol via mixed-acid fermentation and butanediol fermentation (McDonald et al., 1991), the fermentation products were found at low levels in this study. Our data showed that unclassified Enterobacteriaceae were more abundant in the initial ensiling when acetic acid was low. The PCoA and network analyses indicated that unclassified Enterobacteriaceae were negatively related to acetic acid content. Therefore, tropical grass silage may be characterized by a high abundance of Enterobacteriaceae, even without substantial production of ethanol and 2,3-butanediol. In DC silage stored at 40°C that was examined in this study, lactic acid levels did not increase, while acetic acid levels increased when *Lactobacillus* became more abundant during prolonged ensiling. This result is difficult to explain using the current knowledge and understanding.

In addition to *Enterobacter*, many Enterobacteriaceae, such as *Citrobacter*, *Escherichia*, *Klebsiella*, *Morganella*, and *Yersinia*, can be detected in pre-ensiled crops and their silages by DNA-based identification (Ni et al., 2017). Although microbiota analysis sometimes produces unclassified families for gut samples, such a large number of unclassified families is unusual for fermented foods. In addition,

if Enterobacteriaceae were abundant, ethanol and 2,3-butanediol would be detected in large amounts, which was not observed in either DC or WT silage. The MiSeq analysis in this study classified *Escherichia*, *Kosakonia*, and *Morganella*; hence, unclassified Enterobacteriaceae may include taxa other than these three species. Further research is necessary to clarify the role of Enterobacteriaceae in tropical grass ensiling.

Although *Wallemia* has been suggested as a promoting factor for acetic acid fermentation, there is no report that *Wallemia* has a high capacity to produce acetic acid. *Wallemia* is a xerophilic mold that is detectable in air, soil, dried food, and salt (Zalar et al., 2005), and a high abundance of *Wallemia* was observed in the high-moisture DC silage in this study. *Saitozyma* and *Papiliotrema*, which have been shown to suppress acetic acid fermentation by network analysis, are integral components of soil and epiphytic plants (Surussawadee et al., 2014; Vujanovic, 2021). Likewise, *Sporobolomyces* are commonly found in low-nutrition environments (Kot et al., 2021). Information on the fungal microbiota of silage obtained by amplicon sequencing is lacking; hence, the roles of these fungi in forage ensiling are not known.

Distinct differences in the fermentation products due to storage temperature (25 and 40℃) were observed in DC silage. At 25℃, butyric acid increased along with acetic acid production, whereas at 40℃, butyric acid was almost completely suppressed, and acetic acid was reduced to about half of that at 25℃. Usually, silage is more susceptible to clostridial fermentation during warm storage when compared to cold storage (R. E. Muck et al., 2003). In addition, ensiling at a high temperature lowers lactic acid content due to a shift from homolactic to heterolactic fermentation (Bernardes et al., 2018). Meanwhile, ensiling at a substantially high temperature, such as at about 40℃, as examined in this study, could increase lactic acid content in tropical grass silage, which would not be seen in other crop silages such as corn and wheat (Weinberg et al., 2001). Li et al. (D. Li et al., 2019) found higher lactic acid production at 40℃ than at 28℃ in king grass, paspalum, and stylo silages, whereas no differences were found in acetic acid content between the two storage temperatures. Likewise, Gulfam et al. (Gulfam et al., 2017) found that, although acetic acid was predominant in napier grass silage at 30℃, lactic acid became prevalent when stored at 40℃. In this study, storage at 40℃ increased lactic acid fermentation and suppressed acetic and butyric acid fermentation in DC silage, with a marked decrease in *Clostridium* abundance in the bacterial microbiota. In WT silage, lactic acid fermentation was enhanced without a marked change in acetic acid content at 40℃ compared to 25℃. A significant decrease in *Lactococcus* and *Enterococcus*, and an increase in *Bacillus* at high-temperature storage was observed in WT silage. *Bacillus* is generally regarded as a strictly aerobic bacterium, but facultative anaerobic species are also present, and their ability to produce lactic acid has been shown (Ohara & Yahata, 1996; Payot et al., 1999). In WT silage stored at 40℃, lactic acid increased in the presence of *Bacillus* and decreased in the presence of *Lactococcus* and *Enterococcus*. Whether fermentation during high-temperature storage differs between tropical and other forages and whether *Bacillus* helps to promote lactic acid production requires further investigation.

2.4. Summary

Typical acetic acid fermentation was found in DC silage, and *Lactobacillus* and unclassified Enterobacteriaceae were shown to be involved. The relationship between fermentation and fungal microbiota was unclear, although *Wallemia* was shown to be a promoting factor for acetic acid

fermentation. Storage at 40°C suppressed acetic acid fermentation in DC and WT silage, indicating that high storage temperatures may not adversely affect silage fermentation. Regardless of the degree of acetic acid production, guinea grass fermentation appeared to be bacteria-driven and may not be depicted by the fungal microbiota. Mixed fermentation and 2,3-butanediol fermentation were not found, even with a high abundance (>60%) of unclassified Enterobacteriaceae.

CHAPTER 3 Microbiota of Guinea Grass Silage Exhibiting Lactic Acid Fermentation

Microbiota is a pivotal role during ensiling to ensure high-quality and safe forage for intensive ruminant livestock across the year. Guinea grass (*Panicum maximum*), as a tropical grass, has good nutritional value and high biomass production (e.g., 20–30 tons dry matter (DM)/ha) in the tropics and subtropics (Aganga & Tshwenyane, 2004; Daniel et al., 2019). But ensiling of tropical grasses has its challenges, including high moisture (high risk of clostridia deterioration) at the time of cutting and a low water-soluble carbohydrates (needed for desirable fermentation of lactic acid bacteria) compared with corn or sorghum (Santos et al., 2013). Although wilting and molasses addition could overcome these restrictions and enhance the desirable fermentation of the ensiling process, acetic acid is often regarded as the dominant fermentation product in tropical grass silages (Yanbing Li & Nishino, 2013a; Nishino et al., 2012). Occasionally, more product of lactic acid than acetic acid could also be observed in high-moisture (DM, 286 g/kg) guinea grass silage without inoculant or molasses addition (Parvin & Nishino, 2009).

Lactic acid bacteria (LAB), including Lactic acid-producing rod (*Lactobacillus*) and cocci (*Leuconostocs*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Streptococcus*), play a key role for lactic acid fermentation in silage, and heterofermentative process of LAB can produce additional acetic acid. Otherwise, acetic acid could also be produced from the anaerobic metabolism of lactic acid by *Lactobacillus buchneri* and *Lact. hilgardii* (Heinl et al., 2012; Oude Elferink et al., 2001). Moreover, the non-LAB *Enterobacteriaceae* spp. are important in their competition with the LAB flora for substrate duo to the second most numerous bacterial group of the epiphytic microflora in the silo, some of which ferment glucose to a mixture of acids, including acetic acid (Pahlow et al., 2003). Apart from bacteria, a number of yeasts, such as *Candida* spp., *Hansenula* spp., *Saccharomyces* spp. and *Torulopsis* spp., are capable of producing lactic acid, acetic acid and ethanol under anaerobic conditions by fermentation of sugars (McDonald et al., 1991). Thus, characterizing the bacterial and fungal microbiota involved in ensiling process is central to understand the unavoidable acetic acid fermentation in tropical grass silage. Bacterial community has been monitored by culture-independent denaturing gradient gel electrophoresis (DGGE) analysis in the ensiling process for guinea grass, which revealed that the changes of bacterial population were associated with enhanced lactic acid fermentation by wilting and molasses addition (Nishino et al., 2012); and many species of enterobacteria were found in common between the pre-ensiled material and silages of guinea grass (Yanbing Li & Nishino, 2013a). These results described above are encouraging, but it could not help determining which bacteria were associated with enhanced acetic acid fermentation. This may because distinctive differences could not be seen on the bacterial community between short- and long-term ensiling due to the limitation for quantitative and the whole microbiota by DGGE.

In addition, the next-generation sequencing (NGS) of 16S rDNA or internal transcribed spacer (ITS) amplicon has been applied to explore the microbial ecology of silage, which can provide more comprehensive insights into the composition of the complete bacterial and fungal community of interest (McAllister et al., 2018). Numerous studies have evaluated the effects of different inoculants or chemical additives on the microbial community succession involved in fermentation quality and aerobic stability

of whole-plant corn (C. Bai et al., 2021; Benjamim da Silva et al., 2021; Keshri et al., 2018; Y. Zhang et al., 2020), alfalfa (J. Bai et al., 2021) and wheat silages (Keshri et al., 2019). It is now clear that the bacterial populations shifted dramatically from the initial silages, and replaced by one that is adapted to ensiling environment (Ni et al., 2017). Furthermore, more LAB populations, especially a greater abundance of *Lactobacillus* could be observed in inoculated silages, and inoculation of heterofermentative *Lact. buchneri* or *Lact. hilgardii* did affect the fungal community composition by reducing the abundance of *Saccharomycetes* in corn silage (Benjamim da Silva et al., 2021). It was also reported that the addition of salt and sugar would be a promising effective approach to improve the silage quality by reducing the spoilage fungal populations for elephant grass (Vu et al., 2019). However, the microbiota processes described above may have different responses to different crop silages.

Therefore, due to a limited amount of information on the microbiota involve in fermentation process of guinea grass silage, the present study was aimed at clarifying bacterial and fungal communities by NGS of phylogenetic gene markers in guinea grass silage dominated by lactic acid fermentation. In our previous guinea grass silage experiment, intensive acetic acid fermentation with substantial productions of butyric acid and ethanol occurred during ensiling without wilting prepared from the first growth of guinea grass (Hou et al., 2021); in the current study, dominant lactic acid fermentation was observed in unwilted and wilted ensiling prepared from second-growth of guinea grass. The high temperature (40℃) and molasses addition were anticipated to different fermentation profiles during ensiling. We hypothesized that changes of microbiota associated with different fermentation profiles would be helpful to elucidate the puzzling acetic acid fermentation in tropical grass silage.

3.1. Materials and Methods

Silage preparation

The second growth (42 days after first cutting) of guinea grass (*Panicum maximum* cv. Soirukurin; Snow Brand Seed Co., Ltd., Sapporo, Japan) was harvested at the heading stage on 19 September 2019 at the experimental farm of the Faculty of Agriculture of Okayama University. The grass was chopped using a forage cutter at a theoretical length of 13 mm immediately after harvest (direct-cut) and after field wilting (wilted) for 4 h. The chopped grass was added with molasses (molasses: 10 g/kg fresh weight) and without molasses (control), and 300 g of pre-ensiled material was packed in a plastic pouch (Hiryu BN-12; Asahi Kasei Pax, Tokyo, Japan), which was followed by air removal by using a vacuum sealer. Silos were prepared in triplicate and stored at 25 and 40℃ temperature for 2 weeks and 2 months.

Chemical components and fermentation products analyses

The dry matter (DM) contents of pre-ensiled and silage samples were determined at 60℃ for 48 h in a forced-air oven. The pH value, as well as the lactic acid, short-chain fatty acid, alcohol, and ammonia nitrogen contents were determined from the water extracts (Tran Thi Minh et al., 2014). The pH was measured using a glass electrode pH meter. The acids and alcohols were determined using an ion-exclusion polymeric high-performance liquid chromatography method with refractive index detection. A portion of the water extract was passed through a 0.20 μm filter, and 10 μL of the filtrate was injected into an IC-Sep COREGEL-87H column (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The mobile phase used was 0.004 M sulfuric acid, and the flow rate was 0.6 mL/min at 60℃. The ammonia nitrogen content was determined using the phenol–hypochlorite reaction (Broderick & Kang, 1980).

MiSeq preparation

Two silages were chosen at random from triplicate silages and subjected to DNA extraction and subsequent MiSeq analyses (Wali & Nishino, 2020). Frozen samples (5 g) were thawed, and 95 mL of sterilized phosphate-buffered saline (pH 7.4) was added. The samples were shaken vigorously for 10 min and microbial pellets were obtained by centrifugation at 8000× *g* for 15 min. DNA extraction was performed using the repeated bead-beating and column method (Yu & Morrison, 2004), followed by DNA purification using a QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan). Bacterial 16S rRNA genes spanning a hypervariable (V4) region were amplified using the F515 (forward: 5-GTGCCAGCMGCCGCGGTAA-3) and R806 (reverse: 5-GGACTACHVGGGTWTCTAAT-3) primers (Caporaso et al., 2011). Fungal rRNA genes spanning the internal transcribed spacer 2 (ITS2) region were amplified using the gITS7 (forward: 5-GTGAATCATCGARTCTTTG-3') and ITS4 (reverse: 5-TCCTCCGCTTATTGATATGC-3) primers (Ihrmark et al., 2012). A two-round polymerase chain reaction (PCR) was performed to reduce any potential bias of the MiSeq adaptor overhang (Berry et al., 2011). For the V4 region of bacterial 16S rRNA genes, the PCR conditions were as follows: initial denaturation at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 30 s, and a final extension step at 72℃ for 5 min. For the ITS2 region of the fungal rRNA genes, the following conditions were employed: 95℃ for 5 min, followed by 40 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s, and a final extension step at 72℃ for 10 min. The PCR products were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and moved to a second round of PCR with adapter-attached primers. Amplification was performed using the following temperature profiles: one cycle at 94℃ for 2 min, 10 cycles of denaturation at 94℃ for 30 s, annealing at 59℃ for 30 s, extension at 72℃ for 30 s, and one cycle at 72℃ for 5 min for the final extension. The PCR products were purified as described above for the first-round products. The purified amplicons were pair-end sequenced (2 × 250) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan).

Microbial quantification by real-time PCR

Quantitative PCR (qPCR) was carried out in optical-grade 96-well plates on an AriaMx real-time PCR system (Agilent Technologies, Ltd., Tokyo, Japan). For total bacteria, the primers were 16S F (forward: 5′-TCCTACGGGAGGCAGCAGT-3′) and 16S R (reverse: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′) (Peng et al., 2018), and the PCR cycling conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 1 min at 60℃, and 1 min at 72℃. For total fungi, the primers were FF390 (forward: 5'-CGATAACGAACGAGACCT-3') and FR1 (reverse: 5'-AICCATTCAATCGGTAIT-3') (Peng et al., 2018), and the PCR cycling conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 30 s at 50℃, and 30 s at 72℃. The rRNA gene copy numbers quantified by real-time PCR were expressed as log10 copies/g silage.

Bioinformatics analysis

Bioinformatics analysis was performed using the QIIME2 program (Bolyen et al., 2019). The raw paired-end FASTQ reads of bacteria and fungi were demultiplexed using the q2-demux plugin. Primer sequences were removed from the bacterial demultiplexed sequence data for quality control. For fungi, demultiplexed sequence data were trimmed with q2-ITSxpress (Rivers et al., 2018) and an unmerged ITS2 sequence from a fungal amplicon sequencing dataset was extracted for quality control. DADA2 (Callahan et al., 2016) was used to filter, trim, denoise, and merge the data. Chimeric sequences were

removed using the consensus method. For phylogenetic diversity analysis, all observed amplicon sequence variants (Callahan et al., 2017), that is, 100% operational taxonomic units (OTUs), were aligned using the MAFFT program plugin via q2-alignment (Katoh et al., 2002) to construct a phylogenetic tree with FastTree2 via q2-phylogeny (Price et al., 2010). Taxonomic classification was assigned using the SILVA database (version 132), specific for the V3-4 region of 16S rRNA genes, and the UNITE fungal ITS database (version 8.2), specific to the ITS2 region of fungal rRNA genes (Nilsson et al., 2019). All taxonomic classifications were implemented using QIIME2 and assigned using the naïve Bayesian algorithm. The alpha diversity (observed OTUs and Faith's phylogenetic diversity) was estimated using q2-diversity at the OTU level.

Statistical analysis

Data for fermentation products, bacterial and fungal copy numbers, the alpha diversity of bacterial and fungal microbiota, and relative abundances of major bacterial and fungal genera were subjected to a one-way analysis of variance. Duncan's multiple range test was used to determine significant differences between the means at a probability of <0.05. These analyses were performed using IBM SPSS Statistics (Version 26.0, IBM Co., Armonk, NY, USA, 2019)

The beta-diversity of bacterial and fungal microbiota was calculated based on the Bray-Curtis distance, using the filtered genus-level taxa and was visualized using principal coordinate analysis (PCoA) with Primer-E software (version 7, Quest Research Ltd., Auckland, New Zealand). Discriminant vectors with a Pearson correlation >0.7 were considered significant. Correlations between fermentation products and the relative abundance of bacterial and fungal genera were calculated using Spearman's rank correlation coefficient. Significant correlations at a probability of <0.05 between the fermentation products and dominant genera were visualized by network analysis using the Cytoscape software (version 3.8.2) (Shannon et al., 2003).

3.2. Results

Fermentation products

The DM contents of DC and WT guinea grass were 176 and 266 g/kg, respectively. Lactic and acetic acids were not detected in the pre-ensiled materials.

A large amount of lactic acid (more than 45.0 g/kg DM) and a small amount of acetic acid (less than 11.0 g/kg DM) were produced in the DC silage for 2 weeks regardless of molasses addition and storage temperature, and the pH value was also less than 4.90 (Table 3.1). In DC silage, there was no increase in lactic acid content from 2 weeks to 2 months, but the acetic acid content distinctly increased and more than three-fold especially when the control silage was stored at 25℃ and molasses treated silage stored at 40℃. And a significantly higher ammonia nitrogen content was observed in control silage stored at 25℃ for 2 months of DC silage. Obviously high levels of lactic acid content but low levels of acetic acid content, and more than 7.50 ratio of lactic acid to acetic acid content were observed in the WT silage for 2 weeks regardless of molasses addition and storage temperature (Table 3.1). In WT silage, the lactic acid and acetic acid both clearly increased from 2 weeks to 2 months, the pH value reduced to less than 4.80, but the ratio of lactic acid to acetic acid content clearly decreased. A significantly higher ammonia nitrogen content was also observed in control silage stored at 25℃ for 2 months of WT silage.

The effect of molasses on concentrations of lactic and acetic acid was different by different storage temperatures and wilting treatment (Table 3.1). In DC silage after 2 months, the lactic acid content was increased but acetic acid was decreased by molasses addition when ensilaged at 25℃; whereas the lactic acid was not affected but acetic acid was increased by molasses addition when ensilaged at 40℃. In WT silage after 2 months, the concentrations of lactic and acetic acid were both decreased by molasses addition when ensilaged at 25℃; whereas the lactic and acetic acid were both increased by molasses addition when ensilaged at 40℃.

Table 3.1 Dry matter, pH value and fermentation products of direct-cut and wilted guinea grass silage without and with molasses addition stored at 25 and 40℃ for 2 weeks and 2 months.

				DM	pH	Lactic acid	Acetic acid	L/A	Ethanol	Ammonia-N
				g/kg FW		g/kg DM	g/kg DM		g/kg DM	g/kg
DC	25℃	Cont	2W	189b	4.70b	59.1bc	8.93c	6.65a	3.47ab	1.22b
			2M	189b	4.86a	50.9c	32.6a	1.65c	2.90b	2.37a
		Mola	2W	184b	4.36c	76.4a	10.3c	7.41a	3.73ab	1.06b
			2M	190b	4.22c	83.1a	17.1b	4.94b	3.39ab	1.38b
	40℃	Cont	2W	191b	4.88a	48.1c	9.96c	4.81b	4.32a	1.42b
			2M	218a	4.57b	59.0bc	21.0b	2.82c	3.11b	1.42b
		Mola	2W	195b	4.54b	68.5ab	9.48c	7.40a	4.01ab	1.22b
			2M	219a	4.60b	50.9c	31.2a	1.63c	4.34a	1.36b
			SE	6.72	0.05	5.16	2.01	0.39	0.35	0.12
One-way ANOVA: *P* value				<0.01	<0.01	<0.01	<0.01	<0.01	0.071	<0.01
WT	25℃	Cont	2W	257B	5.32BC	34.6B	2.64D	13.1A	5.57	0.66D
			2M	260B	4.71D	45.3A	14.4A	3.15E	3.70	1.67A
		Mola	2W	282B	5.44AB	27.7C	3.60D	7.61C	3.63	0.77CD
			2M	276B	4.54E	36.2B	8.46BC	4.31DE	5.66	1.24B
	40℃	Cont	2W	279B	5.45A	26.7C	2.98D	8.93B	5.94	0.87C
			2M	308A	4.78D	37.8B	7.65C	4.96D	4.59	1.19B
		Mola	2W	280B	5.24C	27.2C	3.45D	7.86BC	4.03	0.87C
			2M	309A	4.75D	45.9A	9.59B	4.79D	5.91	1.14B
			SE	8.38	0.04	2.29	0.39	0.42	0.99	0.04
One-way ANOVA: *P* value				<0.01	<0.01	<0.01	<0.01	<0.01	0.449	<0.01

DM, dry matter; L/A, lactic to acetic acid ratio; SE, Pooled standard error; Values in the same column with different following letters (a–d, A–E) are significantly different.

Microbial rRNA copy numbers

The total bacterial and fungal copy numbers of pre-ensiled DC and WT materials were 7.34 and 5.85, and 7.16 and 5.37, respectively. The total bacterial copy numbers numerically increased after 2 weeks in both DC and WT silages regardless of storage temperature and molasses addition, which decreased after 2 months in molasses treated DC silage stored at 25℃, control of DC silage stored at 40℃, and molasses treated WT silage stored at 40℃ (Table 3.2). The total fungal copy numbers numerically decreased after 2 weeks in DC silage, and WT silage stored 40℃, but in WT silages stored at 25℃ for 2 weeks, it was similar to pre-ensiled WT materials (Table 3.3). The total fungal copy numbers decreased from 2 weeks to 2 months in DC and WT silages regardless of storage temperature and molasses addition. Besides, the total fungal copy numbers was lower when ensilaged at 40℃ compared to 25℃ regardless of wilting and molasses addition.

Bacterial microbiota

A total of 1,677,504 filtered 16S rRNA high-quality sequences were obtained to classify 840 OTUs from 34 samples. The observed OTUs and the Faith PD of pre-ensiled DC and WT materials were 218 and 26.1, and 134 and 19.4, respectively. These two indices were distinctly decreased after ensiling in both DC and WT silages. The observed OTUs significantly increased from 2 weeks to 2 months in DC silage stored at 40℃ regardless of molasses addition (Table 3.2). The Faith PD was not changed from 2 weeks to 2 months in DC and WT silages regardless of storage temperature and molasses addition.

The five most abundant bacterial genera in the pre-ensiled DC crop were *Acinetobacter* (24.6%), *Pantoea* (11.2%), *Paenibacillus* (7.73%), *Sphingomonas* (9.57%), *Bacillus* (5.72%), and unclassified Enterobacteriaceae (4.32%), and those in WT crop were *Pseudomonas* (31.5%), *Acinetobacter* (21.2%), *Pantoea* (14.4%), unclassified Enterobacteriaceae (6.05%), and *Paenibacillus* (5.97%) (Figure 3.1).

Table 3.2 Bacterial copy number, α diversities of bacterial community, and relative abundances (%) of five most abundant bacterial genera of direct-cut and wilted guinea grass silage without and with molasses addition stored at 25 and 40℃ for 2 weeks and 2 months.

				Total bacteria log copies/g silage	Observed OTUs	Faith PD	Lactobacillus	unclassified Enterobacteriaceae	Lactococcus	Enterococcus	Pediococcus
DC	25℃	Cont	2W	8.90ab	64.5ab	8.93	25.8c	19.6bcd	30.4a	11.4b	7.93c
			2M	8.29bcd	74.5a	10.8	50.9a	14.4d	16.3bc	8.18b	6.67c
		Mola	2W	8.95a	53.5bc	8.82	49.3a	18.0cd	16.4bc	5.36b	6.44c
			2M	7.83d	52.0bc	8.11	37.5b	29.7ab	18.7b	4.92b	4.41c
	40℃	Cont	2W	8.51abc	41.0c	6.87	18.6c	28.8abc	13.8bc	29.6a	7.62c
			2M	7.01e	59.5b	10.5	20.5c	20.0bcd	6.37cd	15.3b	35.5ab
		Mola	2W	8.16cd	41.0c	6.51	19.0c	32.3a	16.5bc	15.4b	14.0bc
			2M	7.72d	56.5b	9.22	38.0b	10.5d	3.64d	6.84b	39.6a
			SE	0.19	4.25	1.00	3.12	3.27	2.92	2.96	6.84
One-way ANOVA: *P* value				<0.01	<0.01	0.125	<0.01	<0.05	<0.01	<0.01	<0.05
WT	25℃	Cont	2W	8.99A	62.0	10.2	16.8BC	30.7ABC	28.5A	15.5AB	4.36
			2M	8.97A	47.5	8.44	70.5A	15.9C	7.73D	2.14C	1.62
		Mola	2W	8.80A	72.0	9.68	28.8BC	24.7BC	23.2B	11.4BC	2.79
			2M	8.29AB	69.0	10.1	38.0B	27.1BC	12.0C	11.0BC	3.46
	40℃	Cont	2W	7.59BC	76.0	11.1	8.76C	43.8A	15.3C	25.0A	2.34
			2M	6.87C	82.5	12.5	31.0BC	34.0AB	8.27D	18.3AB	2.67
		Mola	2W	8.23AB	60.0	9.22	21.7BC	38.2AB	13.9C	17.6AB	1.99
			2M	7.04C	67.0	10.7	24.3BC	30.5ABC	12.2C	23.6A	2.13
			SE	0.22	9.05	1.11	6.48	4.39	1.15	3.07	0.65
One-way ANOVA: *P* value				<0.01	0.320	0.395	<0.01	<0.05	<0.01	<0.05	0.208

OTUs, operational taxonomic units; PD, phylogenetic diversity; SE, Pooled standard error; Values in the same column with different following letters (a–d, A–D) are significantly different.

Immediately after ensiling, *Lactobacillus*, unclassified Enterobacteriaceae, *Lactococcus*, *Enterococcus* and *Pediococcus* became the dominant taxon in the guinea grass silage (Table 3.2; Figure 3.1). In DC silage for 2 weeks, the abundance of *Lactobacillus* was higher when molasses treated silage stored at 25℃ compared to 40℃ and control silages, which decreased after 2 months; it increased after 2 months when control silage stored at 25℃ and molasses treated silage stored at 40℃; it didn't change after 2 months when control silage stored at 40℃. The abundance of *Lactobacillus* was decreased by molasses

addition when DC silage stored at 25℃ for 2 months, but it was increased by molasses addition when DC silage stored at 40℃ for 2 months. And it was the most abundant genus in DC silage stored at 25℃ for 2 months regardless of the molasses addition. The abundance of unclassified Enterobacteriaceae was marginally higher in DC silage stored at 40℃ than 25℃ for 2 weeks regardless of molasses addition, which decreased after 2 months. And it was increased by molasses addition when DC silage stored at 25℃ for 2 months. The *Lactococcus* was the most abundant genus in control of DC silage stored at 25℃ for 2 weeks, the abundance of which decreased after 2 months. The abundance of *Lactococcus* was double higher in DC silage stored at 25℃ than 40℃ for 2 months, and it was not affected by molasses addition. The abundance of *Enterococcus* was the highest in control of DC silage stored at 40℃ for 2 weeks, which decreased after 2 months, and it was not affected by molasses addition and storage temperature in DC silage after 2 months. The abundance of *Pediococcus* greatly increased (7.62 and 14.0% to 35.5 and 39.6%) from 2 weeks to 2 months in DC silage stored at 40℃ regardless of molasses addition, and it was the most abundant genus in DC silage stored at 40℃ for 2 months.

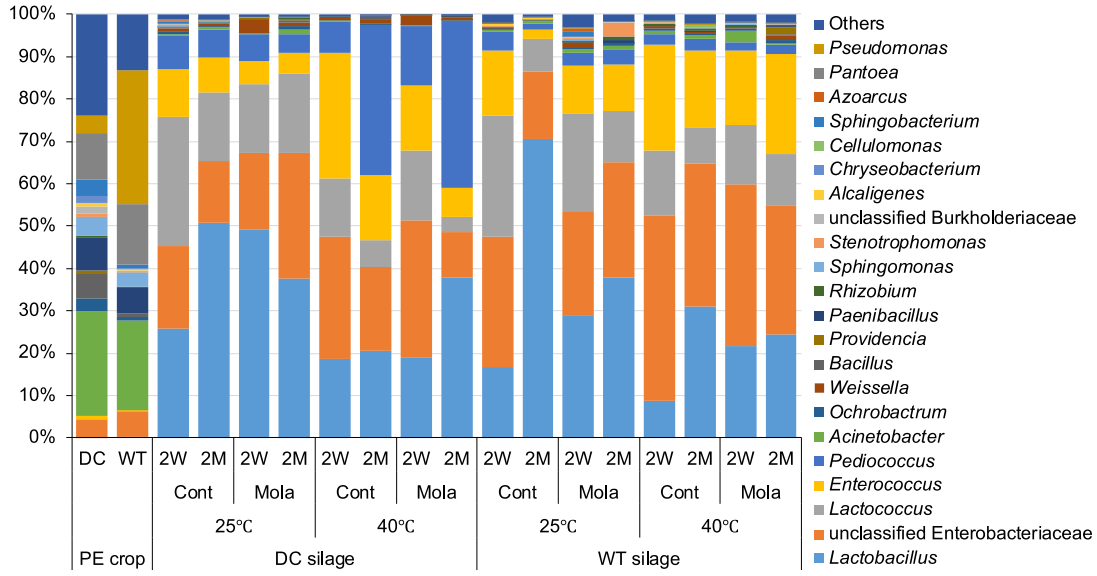

Figure 3.1 Relative abundances of 22 major genera (>1% in abundance) of the bacterial microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. DC and WT indicate direct-cut and wilted crops and silages. Cont and Mola indicate ensiling without and with molasses addition. 2W and 2M indicate storage for 2 weeks and 2 months at 25 and 40℃, respectively.

In WT silage, the abundance of *Lactobacillus* increased from 2 weeks to 2 months and greatly increased (16.8 to 70.5%) especially in control silage stored at 25℃, and it was decreased by molasses addition when ensilaged at 25℃ for 2 months. The abundance of unclassified Enterobacteriaceae was marginally higher in WT silage stored at 40℃ than 25℃, and it was not affected by molasses addition and storage period. The abundance of *Lactococcus* was higher in WT silage stored at 25℃ than 40℃ for 2 weeks regardless of molasses addition, which decreased after 2 months, but it was increased by molasses addition in WT silage for 2 months regardless of storage temperature. The abundance of *Enterococcus* was marginally higher in WT silage stored at 40℃ than 25℃, which was affected by molasses addition, and it decreased from 2 weeks to 2 months in control of WT silage stored at 25℃. The low abundance (less than 4.5%) of *Pediococcus* was observed in WT silage, which was not affected by molasses addition, storage temperature and period at all.

Based on the PCoA plots, pre-ensiled materials were shown to be quite different in the bacterial microbiota from their silages; their microbiota was characterized by *Acinetobacter*, *Pantoea*, *Paenibacillus*, *Pseudomonas*, and others (Figure 3.2). Silage samples were characterized by *Lactobacillus*, *Enterococcus* and unclassified Enterobacteriaceae. And three separate groups were formed for silages, but no separate group was formed by wilting, molasses addition, storage temperature and period.

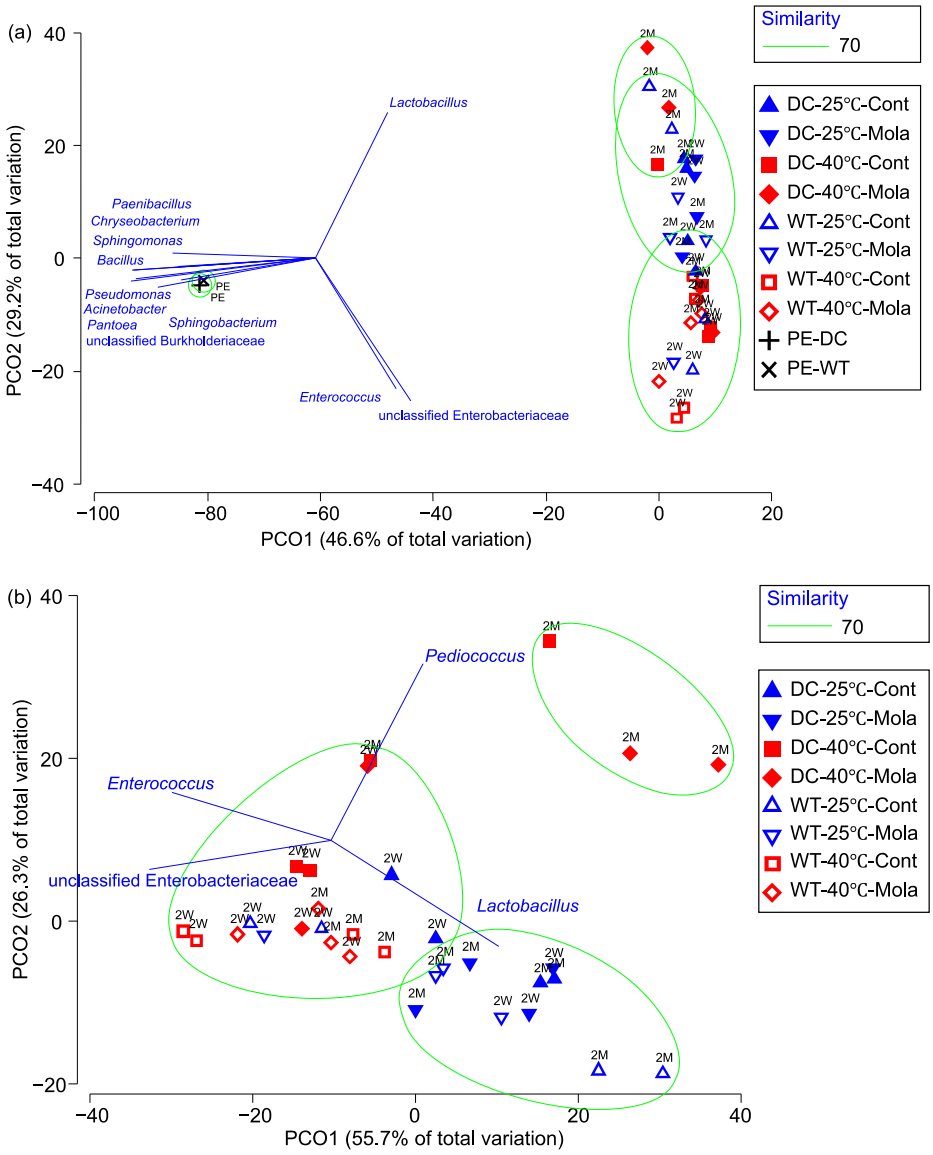

Figure 3.2 Beta diversity of the bacterial microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. The principal coordinate analysis plot (a: all samples including pre-ensiled crops; b: silage samples excluding pre-ensiled crops) of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together on the ordination have a more similar microbiota. The operational taxonomy unit with a Pearson's correlation of >0.7 is overlaid on the plot as vectors. Green circles denote samples enclosed in the same group at a 70% similarity level. PE, DC, WT, Cont, Mola, 2W and 2M indicate pre-ensiled crop, direct-cut silage,

wilted silage, ensiling without and with molasses, storage for 2 weeks and 2 months at 25 and 40℃, respectively.

Fungal microbiota

A total of 167,625 filtered ITS2 high-quality sequences were obtained to classify 714 OTUs. The observed OTUs and Faith PD of pre-ensiled DC and WT materials were 42 and 13.9, and 38 and 14.9, respectively. In DC and WT silages stored at 40℃ for 2 weeks, these two indices numerically decreased, which didn't change after 2 months (Table 3.3). In DC silage for 2 months, these two indices were higher when ensilaged at 25℃ than 40℃ regardless of molasses addition. In WT silage, these two indices were not affected by storage temperature, molasses addition and storage period at all.

The five most abundant fungal genera in the pre-ensiled DC crop were *Saitozyma* (28.8%), *Wallemia* (18.2%), unclassified Ustilaginaceae (11.1%), unclassified Didymellaceae (10.9%), and *Moesziomyces* (8.00%) (Figure 3.3). In DC silage, the abundance of *Wallemia* (38.3-76.4%) numerically increased after 2 weeks, which didn't change after 2 months when ensilaged at 25℃ but decreased after 2 months when ensilaged at 40℃ regardless of molasses addition (Table 3.3). The abundances of *Saitozyma* (7.72-23.1%) numerically decreased after 2 weeks, which didn't change after 2 months regardless of storage temperature and molasses addition.

The five most abundant fungal genera in the pre-ensiled WT crop were *Saitozyma* (42.8%), unclassified Ustilaginaceae (10.7%), *Moesziomyces* (8.20%), *Pseudopithomyces* (5.96%), and *Cladosporium* (5.25%) (Figure 3.3). In WT silage for 2 weeks, the abundance of *Saitozyma* (30.5-46.1%) was higher compared to DC silage, which didn't change after 2 months, regardless of storage temperature and molasses addition. While the *Wallemia* appeared in WT silage even which was not presented in the pre-ensiled WT crop, but the abundance of it was lower compared to DC silage.

Table 3.3 Fungal copy number, α diversities of fungal community, and relative abundances (%) of two most abundant bacterial genera of direct-cut and wilted guinea grass silage without and with molasses addition stored at 25 and 40℃ for 2 weeks and 2 months.

				Total fungi log copies/g silage	Observed OTUs	Faith PD	Wallemia	Saitozyma
DC	25℃	Cont	2W	5.05a	32.0bc	12.6bc	54.4ab	17.1
			2M	4.36b	58.5a	19.4a	57.1ab	6.16
		Mola	2W	4.63b	50.5ab	18.1a	38.3bc	23.1
			2M	3.91c	48.0ab	17.1ab	55.4ab	8.92
	40℃	Cont	2W	4.65b	19.0c	8.91cd	76.4a	7.72
			2M	3.05d	21.0c	9.83cd	19.0cd	22.4
		Mola	2W	3.85c	22.0c	9.12cd	47.1b	13.8
			2M	2.97d	13.5c	6.46d	6.50d	0.00
			SE	0.11	6.99	1.54	7.22	7.90
One-way ANOVA: *P* value				<0.01	<0.05	<0.01	<0.01	0.461
WT	25℃	Cont	2W	5.52A	37.5	14.3	2.72	31.8
			2M	4.43B	72.5	25.4	15.9	22.6
		Mola	2W	5.37A	41.0	14.9	1.49	36.8
			2M	4.16BC	53.5	19.1	1.95	12.9
	40℃	Cont	2W	3.93BCD	28.5	10.7	4.26	46.1
			2M	3.62CD	46.5	16.7	12.5	18.6
		Mola	2W	4.43B	29.0	11.4	5.79	30.5

	2M	3.38D	34.5	13.4	7.87	25.1
	SE	0.18	10.24	2.93	4.62	10.59
One-way ANOVA: *P* value		<0.01	0.168	0.101	0.365	0.497

OTUs, operational taxonomic units; PD, phylogenetic diversity; SE, Pooled standard error; Values in the same column with different following letters (a–d, A–D) are significantly different.

The *Acremonium* and *Myrmecridium* appeared at more than 10.0% abundance in WT silage stored at 25℃, the *Ustilago* appeared at more than 5.00% abundance in WT silage stored at 40℃, and the *Candida* also appeared at more than 10.0% abundance in molasses treated WT silage stored at 40℃ for 2 months, but these fungal genera appeared at scarce abundance (<1%) or disappeared in more than half of silage samples and showed greatly variable abundance among silage samples during ensiling (Figure 3.3).

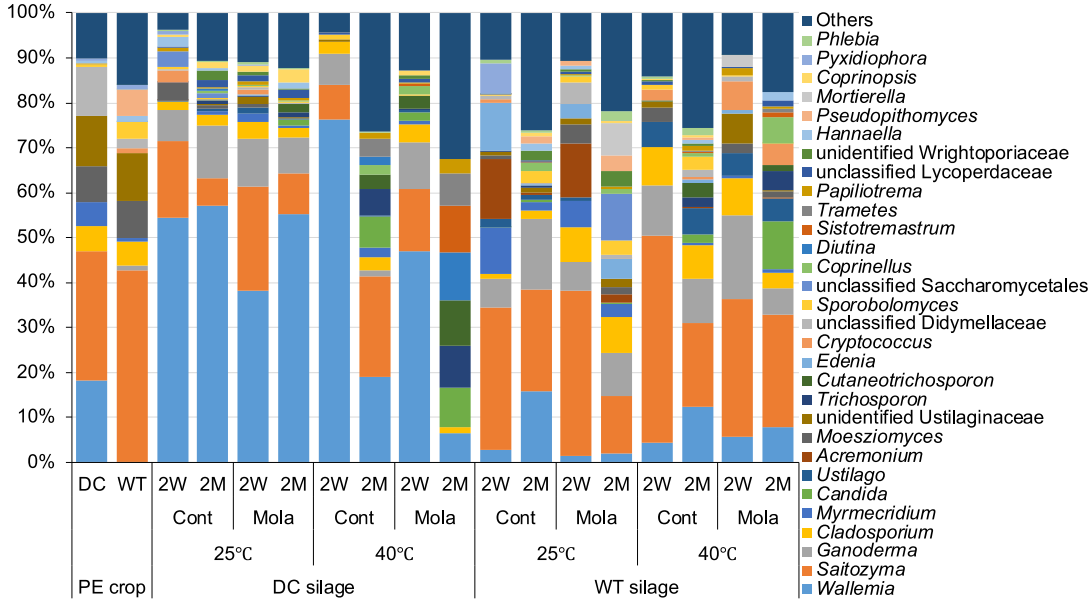

Figure 3.3 Relative abundances of 30 major genera (>1% in abundance) of the fungal microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. DC and WT indicate direct-cut and wilted crops and silages. Cont and Mola indicate ensiling without and with molasses addition. 2W and 2M indicate storage for 2 weeks and 2 months at 25 and 40℃, respectively.

The PCoA plots did not clarify the differences in fungal microbiota between the pre-ensiled crop and WT silages (Figure 3.4). At the 70% similarity level, a separate group was formed for DC silage, which was characterized by *Wallemia*. The *Saitozyma* mainly contributed clusters of pre-ensiled crop and WT silage, but several separate groups were formed. While others emphasized individual silo-to-silo variations.

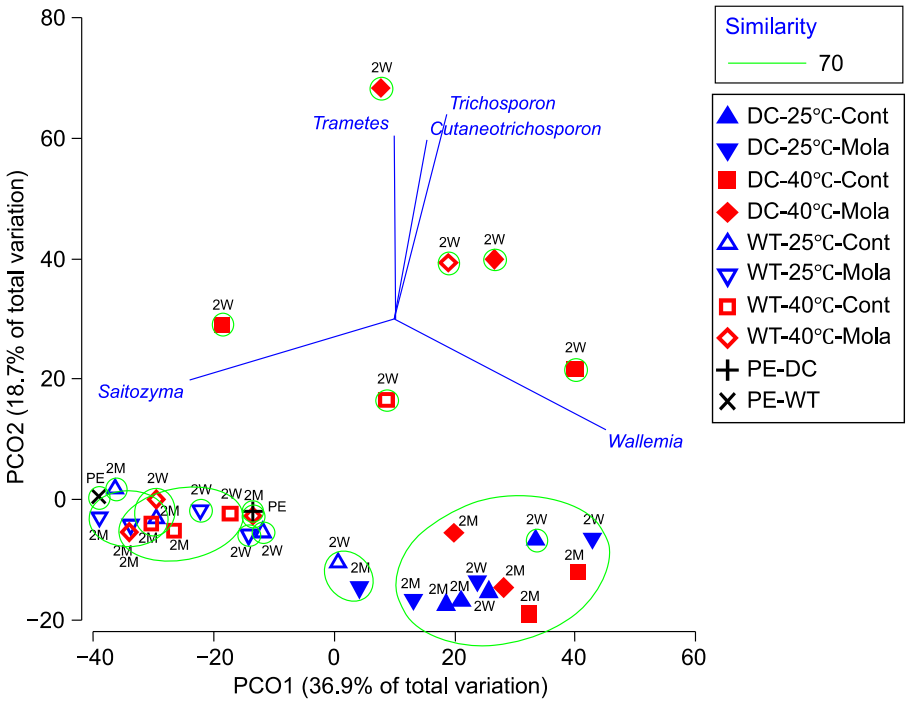

Figure 3.4 Beta diversity of the fungal microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together on the ordination have a more similar microbiota. The operational taxonomy unit with a Pearson's correlation of >0.7 is overlaid on the plot as vectors. Green circles denote samples enclosed in the same group at a 70% similarity level. PE, DC, WT, Cont, Mola, 2W and 2M indicate pre-ensiled crop, direct-cut silage, wilted silage, ensiling without and with molasses, storage for 2 weeks and 2 months at 25 and 40°C, respectively.

Relationships between the fermentation products and core genera of bacteria and fungi

A Spearman's correlation network was generated to illustrate the relationship between fermentation products and dominant microbial genera with an abundance greater than 1% (Figure 3.5). The layout of a circle is sorted by degree centrality. The fermentation product of acetic acid was the key parameter that interacted with others based on the highest degree (15) centrality of all fermentation products and microbial genera, followed by lactic acid (13) and ammonia (11). The unclassified *Enterobacteriaceae* and *Pediococcus* was the key bacteria that interacted with others based on the highest (10) degree centrality of all bacterial genera. The *Wallemia* was the key fungi that interacted with others based on the highest degree (9) centrality of all fungal genera.

Fermentation products of acetic acid, lactic acid and ammonia were positively correlative with each other. The concentration of acetic acid was positively correlative with *Lactobacillus*, *Pediococcus* and *Wallemia*, while negatively correlative with unclassified *Enterobacteriaceae*, *Acinetobacter*, *Ochrobactrum*, *Enterococcus*, *Lactococcus*, *Ustilago*, *Cladosporium*, *Acremonium, Saitozyma* and *Moesziomyces*. The concentration of lactic acid was positively correlative with *Pediococcus, Weissella* and *Wallemia*, while negatively correlative with unclassified *Enterobacteriaceae*, *Acinetobacter*, *Ochrobactrum*, *Enterococcus*, *Cladosporium, Acremonium, Saitozyma* and *Moesziomyces*. The concentration of ammonia was positively correlative with *Lactobacillus* and *Wallemia*, while negatively correlative with unclassified *Enterobacteriaceae*, *Acinetobacter*, *Lactococcus*, *Ustilago, Cladosporium,*

Saitozyma and *Moesziomyces*. The concentration of ethanol was positively correlative with *Enterococcus*, while negatively correlative with *Wallemia*.

The bacterial genus of unclassified *Enterobacteriaceae* was positively correlative with *Enterococcus*, *Acinetobacter*, *Ochrobactrum, Ustilago* and *Cladosporium,* while negatively correlative with *Lactobacillus and Pediococcus*. A negative relationship between *Lactobacillus* and *Enterococcus* was observed. The fungal genus of *Wallemia* was positively correlative with *Weissella*, while negatively correlative with *Saitozyma*, *Moesziomyces*, *Acremonium* and *Myrmecridium*. The fungal genus of *Saitozyma* and *Moesziomyces* were positively correlative with each other, while the *Acremonium* and *Myrmecridium* were also positively correlative with each other.

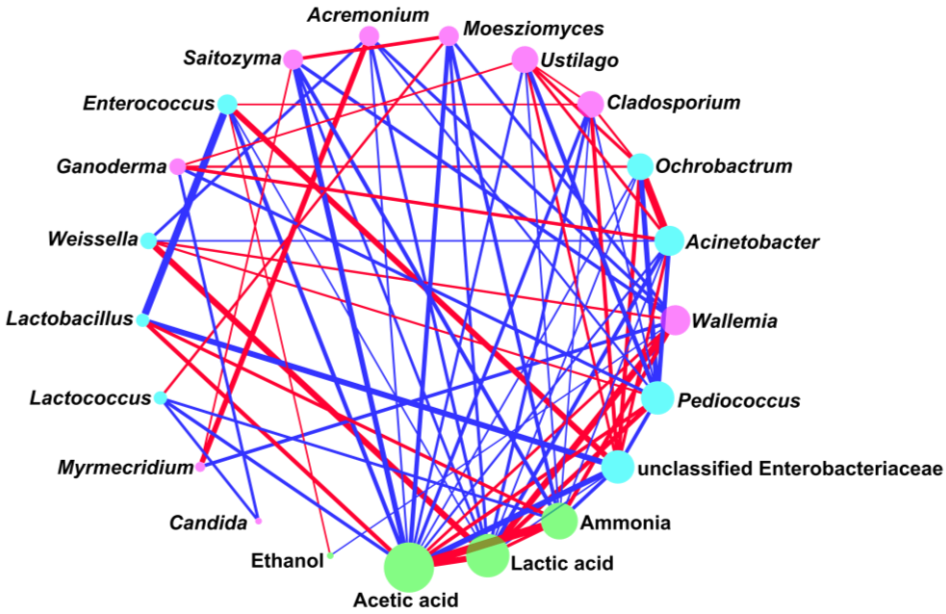

Figure 3.5 Correlation network diagram for fermentation products and major bacterial and fungal genera of direct-cut and wilted guinea grass silage. Green, blue, and purple nodes represent the fermentation product, bacterial genus, and fungal genus, respectively. The node size represents the degree of centrality, which is calculated as the number of edges connecting a node to others. The Spearman's Rho of two nodes is indicated by the edge width, with red and blue edges describing the positive and negative relationships between the two nodes.

3.3. Discussion

Intensive acetic acid fermentation was often observed in DC ensiling of guinea grass without any additive (Li & Nishino, 2013b; Nishino et al., 2012). Surprisingly, in our study, enhanced lactic acid fermentation was observed because high level of lactic acid content (40.5~68.2 g/kg DM) and low level of acetic acid content (17.3~40.4 g/kg DM) even in DC silages without molasses addition after 2 months. However, the ratio of lactic acid to acetic acid was decreased from 2 weeks to 2 months fermentation in all silages, which was increased or not changed during ensiling of corn or barley even showing similar high level of lactic acid production with present study (Guan et al., 2020; B. Liu et al., 2019). The observations here is consistent with this trend, decreasing of the ratio of lactic acid to acetic acid during ensiling of wilted guinea grass (Nishino et al., 2012; Li & Nishino, 2013a). Interestingly, great increasing in acetic acid content with numerical decreasing in lactic acid content was observed in DC silages without molasses addition when stored at 25℃ rather than 40℃, and similar change pattern was also observed in DC silages treated with molasses addition when stored at 40℃ rather than 25℃, which indicate that

acetic acid would be enhanced by persistently moderate (25℃) temperature in DC silages without molasses, but acetic acid would be enhanced by persistently high (40℃) temperature in DC silages with molasses. This difference might be due to more sucrose provided by molasses addition, which would affect acetic acid production by different bacteria at moderate or high storage temperatures. A more heterolactic fermentation would be exhibited for corn silages in warm climates or at higher temperatures (Bernardes et al., 2018), but even so only the heterolactic fermentation could not explain that only acetic acid content was greatly increased without increased lactic acid content in the present study. Wilting is essential for tropical grass ensiling to accelerate lactic acid production and ensure DM recovery, and the lactic acid to acetic acid ratio increased with wilting reported by a previous study (Nishino et al., 2012). Our results also showed less acetic acid content and a high ratio of lactic acid to acetic acid in WT silages. Moreover, increases in productions of lactic acid and acetic acid were consistently observed in WT silages, but the ratio of lactic acid to acetic acid was decreased from 2 weeks to 2 months fermentation even in molasses treated ensiling, which is in agreement with the addition of molasses that could not reverse the decline in the ratio of lactic acid to acetic acid during the ensiling duration of wilted guinea grass silage (Nishino et al., 2012). Thus, it is confirmed that sugar deficiency in the pre-ensiled grass may not be the critical factor for acetic acid fermentation in tropical grass ensiling.

The bacterial diversity and community compositions could influence the fermentation process, some of which are directly related to the fermentation products during ensiling of guinea grass. The highest bacterial richness and diversity was observed in DC silages without molasses addition when ensilaged at moderate (25℃) temperature after 2 months, which was also observed in DC silages treated with molasses addition when stored at high (40℃) temperature. The great increase of acetic acid with a decrease of lactic acid may be associated with this high bacterial richness and diversity during ensiling, because the high level of acetic acid and low level of acetic acid resulted in high pH values that could activate a variety of bacteria. On the other hand, the bacterial richness and diversity were not affected by storage period and temperature in WT silages regardless of molasses treatment, in which there were not much increase in acetic acid content and no decrease in lactic acid content. Pre-ensiling materials had high bacterial richness and diversity. After ensiling of 2 weeks, the richness and diversity was decreased rapidly in all silages, which was similar with Napier grass (Dong et al., 2020), corn and alfalfa silages (Ni et al., 2017).

It is well known that the majority of the bacterial populations involved in lactic acid fermentation in silage belong to the *Firmicutes* phylum (Pahlow et al., 2003; Cai et al., 1998). *Proteobacteria* was the most abundant phylum before ensiling, which was decreased after ensiling but still more than 10% in most silage samples, while *Firmicutes* was increased to an average of 66.9% and 73.9% in 2 weeks and 2 months silages, respectively. It was reported that mature silages of corn, oat and alfalfa were dominated by *Firmicutes* (>97%) (Keshri et al., 2018; Romero et al., 2017; Yang et al., 2019), which is inconsistent with still high abundance of *Proteobacteria* presented in 2 weeks and 2 months silages of our study. The high abundance of *Enterobacteriaceae* resulted in abundant *Proteobacteria* in the present study.

The dominant bacterial genera were unclassified *Enterobacteriaceae*, *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Pediococcus*. Enterobacteria are usually the second most numerous bacterial group of the epiphytic microflora active in the silo and thus the most important in their competition with the LAB flora, producing primarily acetic acid (Pahlow et al., 2003). The abundance of unclassified

Enterobacteriaceae was increased to 26.1% after ensiling from 5.19% in pre-ensilaged materials. However, the abundance of unclassified Enterobacteriaceae might not be associated with lactic acid and acetic acid fermentation in this study, because it was not changed or decreased when greatly increased acetic acid content was observed in DC silages, and it was not changed or decreased when increased concentrations of lactic acid and acetic acid was observed in WT silages. A similar result was also found in our previous guinea grass silage (Hou et al., 2021), which showed intensive acetic acid fermentation during ensiling. Besides, enterobacteria are generally rather sensitive to fast acidification and low pH, and their numbers in silage usually decline sharply when the pH falls below pH 4.5 (Pahlow et al., 2003). It was also reported that the abundances of *Enterobacter* and unclassified Enterobacteriaceae were much lowered by inoculation in alfalfa silage (Su et al., 2019). However, molasses addition didn't affect the abundance of unclassified Enterobacteriaceae at all even the lactic acid fermentation was enhanced by molasses when DC silage stored at 25℃. The function of unclassified Enterobacteriaceae present in this study might be different with deteriorative enterobacteria in alfalfa silage, as discussed above. Moreover, unclassified Enterobacteriaceae was the key bacteria interacted with fermentation products and other microbiota, and it was negatively correlated with fermentation productions of lactic acid, acetic acid and ammonia, and bacterial genera of *Lactobacillus* and *Pediococcus* based on network analysis. Thus, it could be confirmed that acetic acid fermentation was not contributed by unclassified Enterobacteriaceae in guinea grass ensiling, but the appearance of it may negatively affect acetic acid production by interacting with *Lactobacillus* or *Pediococcus*. Nevertheless, further research is needed on the mechanism of this interaction between *Enterobacteriaceae* spp. and LAB during ensiling of tropical grass.

The LAB genera of *Lactobacillus* (0.07%), *Lactococcus* (0.03%), *Enterococcus* (0.59%) and *Pediococcus* (<0.01%) were found at very low abundance in pre-ensilaged guinea grass. These LAB were increased to dominant bacteria in silage after 2 weeks and 2 months of ensiling, which is consistent with the similar shift from much low abundance of LAB in fresh forage to the dominance of LAB in terminal corn, small grain or legume silage (Mogodiniyai Kasmaei et al., 2017; Duniere et al., 2017; McAllister et al., 2018). The abundance of *Lactobacillus* was not the only predominant bacterial genus after ensiling of guinea grass even showing a high level of lactic acid production in this study, which is different from the predominant *Lactobacillus* genus, encompassing more than 90% of abundance in terminal corn silage (Keshri et al., 2018; Ni et al., 2017). The more diverse LAB populations observed in guinea grass ensiling agree with the abundant genera of *Enterococcus*, *Pediococcus* or *Lactococcus* shown in alfalfa and purple prairie clover silages (Ni et al., 2017; Yang et al., 2019; Peng et al., 2018).

The abundance of *Lactobacillus* was highly changeable from 2 weeks to 2 months during ensiling, but the increased *Lactobacillus* did not enhance much production of lactic acid during ensiling process and there was no correlation between the abundance of *Lactobacillus* genus and lactic acid content by Network analysis, which suggest that the homolactic fermentation of *Lactobacillus* spp. might be limited especially after 2 weeks ensiling of guinea grass. And the metabolism within *Lactobacillus* spp. was different, such as some species of *Lactobacillus* active in homofermentative, some species of Lactobacillus active in heterofermentative, and some species of *Lactobacillus* active in lactic acid-degradation metabolism. These differences within *Lactobacillus* spp. may result in no clear relationship between lactic acid product and genus of *Lactobacillus*. This is in agreement with our previous guinea grass study showing that the abundance of *Lactobacillus* was increased but no increase in production of

lactic acid from 3-day to 1-month of ensiling (Hou & Nishino, 2021). Interestingly, the increased production of acetic acid may be account for increased *Lactobacillus* spp., because the great increase in acetic acid content was observed in DC silages during ensiling process, which also showed clearly increased abundance of *Lactobacillus*. It is known that acetic acid, 1,2-propanediol and traces of ethanol could be produced from the anaerobic metabolism of lactic acid by *Lactobacillus* sp. in silage fermentation (Oude Elferink et al., 2001). But it is difficult to explain that acetic acid was mainly attributed to the lactic acid-degrading activity of *Lactobacillus* spp. during ensiling of guinea grass, because there was no clear decrease in lactic acid content along with the clear increased acetic acid content from 2 weeks to 2 months, and 1,2-propanediol was not detected after 2 months ensiling in the present study. Otherwise, the strain of *Lactobacillus* sp. had genes encoding two critical enzymes (endo-β-1,4-xylanase and β-xylosidase) required for the degradation of xylan, and was able to grow in medium with xylan (and xylose) as the sole carbon source and synthesized higher amounts of acetic acid (Kupryś-Caruk et al., 2021). This may also be one of the reasons why *Lactobacillus* spp. could associate high acetic acid product in guinea grass duo to high hemicellulose component in cell walls of tropical grass. Moreover, the abundance of *Lactobacillus* was positively correlated with concentrations of acetic acid and ammonia based on network analysis. The production of ammonia-N may also be contributed by *Lactobacillus* spp, because increased ammonia-N content along with an increased abundance of *Lactobacillus* was observed from 2 weeks to 2 months in this study, and ammonia can be produced by amino acid deamination by certain LAB in silages (S. Q. Liu et al., 2003; Nishino et al., 2012).

The abundance of *Lactococcus* and *Enterococcus* were decreased or unchanged but clear increase in acetic acid content was observed from 2 weeks to 2 months, which could demonstrate that acetic acid fermentation was not attributed to *Lactococcus* spp. or *Enterococcus* spp.. Alternatively, the predominant genera of *Lactococcus* and *Enterococcus* may highly contribute to lactic acid production during the early stage of guinea grass ensiling, because these two genera, as homofermentative cocci-shaped LAB, usually initiate lactic acid fermentation in silages, creating a suitable environment for further ensiling process (Cai et al., 1998). It was also reported that the higher abundance of *Lactococcus* is related to higher lactic acid content in whole crop corn or napier grass silages (Mudasir Nazar et al., 2020)(M Nazar et al., 2020). Strikingly, *Pediococcus* was the most dominant genus after 2 months in DC silage when stored at 40℃, but it is difficult to state that *Pediococcus* spp., as a group of homo-fermentative LAB, could contribute to acetic acid fermentation, as much increased acetic acid content with less abundance of *Pediococcus* during ensiling was observed in the present study. Moreover, *Pediococcus* spp. could ferment xylose as a carbohydrate source and could be grown at high temperatures (45℃ and 50℃) (Cai et al., 1999), which maybe support the abundant *Pediococcus* spp. observed in high temperature stored ensiling of DC guinea grass. Furthermore, there was no clear increase in abundance of *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Pediococcus* duo to wilting or molasses addition, although *Pediococcus pentosaceus* and *Lactococcus garvieae* were detected by DGGE when lactic acid fermentation was enhanced by wilting and molasses addition in guinea grass ensiling (Nishino et al., 2012) and the *Enterococcus* spp. have a preference for a low-moisture environment (Ni et al., 2017). It could partially explain that lactic acid fermentation was predominant both in DC and WT ensiling.

In contrast to these measures for the bacterial community, the richness and diversity of the fungal community were not clearly reduced in post-ensilaged samples compared with pre-ensiling materials. Alternatively, there were no genera dominating the fungal community after ensiling, which suggested

that the population of fungi was more variable and certain fungi could not succeed after ensiling of guinea grass. Similar results were also observed in our previous guinea grass study even showed different fermentation processes (Hou & Nishino, 2021) . However, it was reported that the fungal richness and diversity decreased in whole-crop corn silages with or without inoculant (Keshri et al., 2018), whereas which increased in alfalfa and elephant grass silages under natural conditions (J. Bai et al., 2020)(Vu et al., 2019). Thus, the fungal community of tropical grass silage may be more diverse than the bacterial community. In addition, the richness and diversity of the fungal community were decreased by high temperature (40℃) in DC silages regardless of storage period and molasses addition, which may result from the high abundance of *Pediococcus* in DC silages when stored at high temperature. *Pediococcus* spp. were found to produce unique broad-spectrum antifungal compounds that had antifungal activity against a number of common spoilage mold and yeast (Magnusson et al., 2003; Mandal et al., 2013).

Basidiomycota was the predominant phylum in both DC and WT guinea grass before ensiling, with an abundance of 68.5 and 70.6%, respectively. The *Basidiomycota* abundance increased and *Ascomycota* abundance decreased after 2 weeks ensiling especially in DC silages, and there was no clear change for these two phyla after 2 months ensiling, which is clearly different from previous studies that the most dominant *Ascomycota* phylum in corn or oat silages, using non-NGS identification techniques or NGS approach (Dolci et al., 2011; Keshri et al., 2018; Romero et al., 2017), although it was also reported that *Basidiomycota* was the dominant phylum in barley silages at the early stage of ensiling (B. Liu et al., 2019).

Genera of *Wallemia* and *Saitozyma*, belonging to phylum of *Basidiomycota*, were both prevalent in pre-ensiling materials and silages. *Wallemia* spp. are xerophilic mold of the phylum *Basidiomycota*, which is known from air, soil, dried food (causing spoilage) and salt (Zalar et al., 2005). The genera of *Saitozyma*, as yeast in the order Tremellales, are integral components of fungal communities in soil and epiphytic plant (Aliyu et al., 2019; Vujanovic, 2021). *Wallemia* may be associated with fermentation of lactic and acetic acid since the higher abundance of which was observed in DC silages, with higher lactic and acetic acid contents than in WT silages. On the contrary, *Saitozyma* was showed the higher abundance in WT silages than DC silages, which indicated that the yeast of which may be associated with suppressed ensiling of guinea grass. Moreover, the fermentation products of lactic and acetic acid were consistently positively correlated with the abundance of *Wallemia*, while negatively correlated with the abundance of *Saitozyma* were detected by Network analysis. These two genera were also found in our previous study of guinea grass silage (Hou & Nishino, 2021), but few studies reported the role of which in other type silages.

The genera of *Cyberlindnera* and *Geotrichum*, belonging to the order *Saccharomycetales*, were reported to be abundant in fresh elephant grass, while after ensiling of 15 and 60 days, the putrefactive organisms of *Fusarium*, *Penicillium, Pseudozyma*, *Botrytis* and *Alternaria* were prevalent (Vu et al., 2019). However, fungi belonging to the order *Saccharomycetales* did not appear in pre-ensiling materials, and these putrefactive organisms were also not apparent or very less abundance (<1%) after ensiling of 2 weeks and 2 months in our study. Otherwise, *Cladosporium*, as a filamentous fungal genus, was observed in pre-ensiling materials and all silages even showed low abundance (1.19~8.51%). It was reported that *Cladosporium* spp. could be isolated from forage before ensiling (Spadaro et al., 2015), and the abundance of *Cladosporium* could also be dominant during ensiling of barley (B. Liu et al., 2019),

sugarcane (L. Zhang et al., 2019) or corn (Keshri et al., 2018). Genera of *Moesziomyces* and unclassified *Ustillaginaceae*, belonging to the family *Ustillaginaceae* (order *Ustilaginales*), were showed similarly high abundance in pre-ensiling materials, but decreased as minor basidiomycetous yeasts after ensiling. *Moesziomyces* spp. are mainly isolated from plant surfaces and this and other yeasts from the *Ustilaginaceae* family with the ability to produce cellulase-free xylanase when grown in xylan (Y. Liu et al., 2019; Faria et al., 2019). Further studies are needed to determine whether the appearance of *Ustilaginaceae* spp. can be associated with the high hemicellulose components in cell walls of guinea grass. Interestingly, more than 10% abundance of *Ganoderma*, a genus of polypore fungi in the family *Ganodermataceae*, was first found in silage, many species of which from tropical regions. The order *Saccharomycetales* including *Candida*, *Issatchenkia*, *Kazachstania*, *Pichia* and *Saccharomyces*, as a group of lactic acid-assimilating yeasts, were the most commonly detected fungi associated with terminal silage and can initiate the spoilage process of corn, barley, oat or sugarcane (Pahlow et al., 2003; Duniere et al., 2017; B. Liu et al., 2019; L. Zhang et al., 2019; Benjamim da Silva et al., 2021). However, the genera of *Candida*, *Diutina* and unclassified *Saccharomycetales* were appeared at varying abundance in scarce samples after ensiling. *Diutina,* as a new genus of order *Saccharomycetales* (Khunnamwong et al., 2015), was first found in silage. The different dominant genera found in our study may result from the difference in the original epiphytic population of fungi in the different grasses, or even differences in the soil fungal community, owing to possible contamination of plant material by soil during harvest (Keshri et al., 2018). Furthermore, the pH value and fermentation products were stable and no visual apparent molds and yeasts after 7 days aerobic exposure for 2 months silage, which suggests that the abundant *Saitozyma* spp. and *Wallemia* spp. probably could not result in aerobic spoilage of mature guinea grass silage, and appeared yeast of *Candida* spp. and *Diutina* spp. would not be activated after aerobic exposure.

3.4. Summary

The predominant lactic acid fermentation was found during ensiling of DC and WT guinea grass regardless of molasses addition or storage temperature. The bacterial genera of unclassified *Enterobacteriaceae*, *Lactobacillus*, *Lactococcus*, *Enterococcus* and *Pediococcus* were dominant in silage after 2 weeks and 2 months of fermentation. The great increased acetic acid content along with clear increased abundance of *Lactobacillus* was observed from 2 weeks to 2 months in DC ensiling. The abundance of unclassified *Enterobacteriaceae*, *Lactococcus* and *Enterococcus* were decreased or unchanged but clearly increased acetic acid content was observed from 2 weeks to 2 months in DC or WT ensiling. Moreover, the unclassified *Enterobacteriaceae* was negatively correlated with fermentation productions of lactic acid, acetic acid and ammonia, and genera of *Lactobacillus* and *Pediococcus*. The fungal population was more variable than bacteria after 2 weeks and 2 months ensiling. The *Wallemia* was showed a higher abundance in DC ensiling with a high level of lactic and acetic acid contents, whereas the *Saitozyma* was shown a higher abundance in WT ensiling with a low level of lactic and acetic acid contents. And the fermentation products of lactic and acetic acid were consistently positively correlated with the abundance of *Wallemia*, while negatively correlated with the abundance of *Saitozyma*. Therefore, the lactic acid fermentation of guinea grass silage could be obtained by the sustained activity of *Pediococcus*, *Lactococcus*, and *Enterococcus* rather than the suppression of *Lactobacillus*.

CHAPTER 4 Microbiota of Whole Crop Corn Silage Produced in a Bunker Silo

In large dairy applications, forage is often ensilaged in bunker silos, which is convenient for machinery loading and suitable for quantity requirements of silage. Silage fermentation is affected by gaseous conditions in the bunker silos varied with spatial position and temporal utilization, because of the highly variable density (less dense near the top than bottom; denser at the center than a wall) during filling, storage and feed-out. Along with changes of fermentation products in bunker silo, the microbial activities would also be affected by these differences because silage production is a completely microbial-based fermentation process. Otherwise, microorganisms from bunker silos may impact animal health and the hygienic quality of dairy farms because of the risk of pathogens and mycotoxins appearing in spoiled and spoiling silages. To our knowledge, few studies have comprehensively assessed the fungal community composition of bunker silo. Especially in the long-term-stored bunker silo (more than one year), unavoidable air penetration to the top layer of bunker silo or to the feed-out facet may cause the growth of fungi.

Along with changes of fermentation products in bunker silo, the microbial activities would also be affected by these differences because silage production is a completely microbial-based fermentation process (R. Muck, 2013). Otherwise, microorganisms from bunker silos may impact animal health and hygienic quality of dairy farms because the risk of pathogens (e.g., *Listeria monocytogenes* and *Clostridium botulinum*) and mycotoxins may appear in spoiled and spoiling silages (Pahlow et al., 2003). Previous studies monitoring microbial communities were carried out in laboratory silos (McAllister et al., 2018; Ni et al., 2017), which may not necessarily portray processes in large-scale silage production facilities because imperfect sealing conditions in large-scale silos are more challenging to proper conservation (Mari et al., 2009; Queiroz et al., 2012). It was reported that analysis of bacterial community characterized by denaturing gradient gel electrophoresis (DGGE) can help understand how diverse non-LAB and LAB species are involved in the ensiling process of bunker-silo (Y Li & Nishino, 2011). Similar with bench-scale model ensiling systems, well-preserved (high concentration of lactic acid and pH values below 5) silage situated in the center was dominated (>90 %) by *Lactobacillus* spp. and low diversity, whereas spoiled (low concentration of lactic acid and high pH values) silage situated in the corners had highly diverse microbiomes with low abundances of *Lactobacillus* spp. (<5 %) (Kraut-Cohen et al., 2016).

Therefore, a spatiotemporal understanding of how bacterial and fungal changes involve in the fermentation process within bunker silos are expected to provide further insights into silage management and fermentation control.

4.1. Sampling and Methods

Silage samples were collected from five dairy farms (hereafter referred to as bunkers A, B, C, D and E) on 6th November 2020 and 26th January 2021. The fiver farms were arranged for a contractor to produce whole-crop corn silage using bunker silos. They used corn harvesters equipped with an on-board kernel processor and a commercial LAB inoculant consisting of *Enterococcus faecium*, *Lactobacillus*

plantarum and *Lact. buchneri.* Six samples were collected from each silo at 0.5 m below the top and 0.5 m above the bottom (Figure 4.1, locations 1, 2, 3, 4, 5 and 6). Approximately 0.5 kg silage samples were collected by digging the silo's feed-out facet to approximately 0.2 m depth.

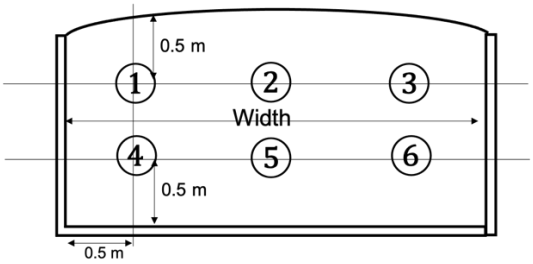

Figure 4.1 Sampling sites in the bunker silos.

Chemical component and fermentation products analyses

The dry matter (DM) contents of pre-ensiled and silage samples were determined at 60℃ for 48 h in a forced-air oven. The pH value, as well as the lactic acid, short-chain fatty acid, alcohol, and ammonia nitrogen contents were determined from the water extracts (Tran Thi Minh et al., 2014). The pH was measured using a glass electrode pH meter. The acids and alcohols were determined using an ion-exclusion polymeric high-performance liquid chromatography method with refractive index detection. A portion of the water extract was passed through a 0.20 μm filter, and 10 μL of the filtrate was injected into an IC-Sep COREGEL-87H column (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The mobile phase used was 0.004 M sulfuric acid, and the flow rate was 0.6 mL/min at 60℃. The ammonia nitrogen content was determined using the phenol–hypochlorite reaction (Broderick & Kang, 1980).

MiSeq preparation

Two silages were chosen at random from triplicate silages and subjected to DNA extraction and subsequent MiSeq analyses (Wali & Nishino, 2020). Frozen samples (5 g) were thawed, and 95 mL of sterilized phosphate-buffered saline (pH 7.4) was added. The samples were shaken vigorously for 10 min and microbial pellets were obtained by centrifugation at 8000× *g* for 15 min. DNA extraction was performed using the repeated bead-beating and column method (Yu & Morrison, 2004), followed by DNA purification using a QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan). Bacterial 16S rRNA genes spanning a hypervariable (V4) region were amplified using the F515 (forward: 5-GTGCCAGCMGCCGCGGTAA-3) and R806 (reverse: 5-GGACTACHVGGGTWTCTAAT-3) primers (Caporaso et al., 2011). Fungal rRNA genes spanning the internal transcribed spacer 2 (ITS2) region were amplified using the gITS7 (forward: 5-GTGAATCATCGARTCTTTG-3') and ITS4 (reverse: 5-TCCTCCGCTTATTGATATGC-3) primers (Ihrmark et al., 2012). A two-round polymerase chain reaction (PCR) was performed to reduce any potential bias of the MiSeq adaptor overhang (Berry et al., 2011). For the V4 region of bacterial 16S rRNA genes, the PCR conditions were as follows: initial denaturation at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 s, 50℃ for 30 s, and 72℃ for 30 s, and a final extension step at 72℃ for 5 min. For the ITS2 region of the fungal rRNA genes, the following conditions were employed: 95℃ for 5 min, followed by 40 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s, and a final extension step at 72℃ for 10 min. The PCR products were purified using the Fast Gene Gel/PCR Extraction Kit (NIPPON Genetics Co., Ltd., Tokyo, Japan) and moved to a second round of PCR with adapter-attached primers. Amplification was performed using the following temperature profiles: one cycle at 94℃ for 2 min, 10 cycles of denaturation at 94℃ for 30 s, annealing

at 59℃ for 30 s, extension at 72℃ for 30 s, and one cycle at 72℃ for 5 min for the final extension. The PCR products were purified as described above for the first-round products. The purified amplicons were pair-end sequenced (2 × 250) on an Illumina MiSeq platform at FASMAC Co., Ltd. (Kanagawa, Japan).

Microbial quantification by real-time PCR

Quantitative PCR (qPCR) was carried out in optical-grade 96-well plates on an AriaMx real-time PCR (RT-PCR) system (Agilent Technologies, Ltd., Tokyo, Japan). For total bacteria, the primers were 16S F (forward: 5′-TCCTACGGGAGGCAGCAGT-3′) and 16S R (reverse: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′) (Peng et al., 2018), and the PCR cycling conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 1 min at 60℃, and 1 min at 72℃. For total fungi, the primers were FF390 (forward: 5'-CGATAACGAACGAGACCT-3') and FR1 (reverse: 5'-AICCATTCAATCGGTAIT-3') (Peng et al., 2018), and the PCR cycling conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 s at 95℃, 30 s at 50℃, and 30 s at 72℃. The rRNA gene copy numbers quantified by real-time PCR were expressed as log10 copies/g silage.

Bioinformatics analysis

Bioinformatics analysis was performed using the QIIME2 program (Bolyen et al., 2019). The raw paired-end FASTQ reads of bacteria and fungi were demultiplexed using the q2-demux plugin. Primer sequences were removed from the bacterial demultiplexed sequence data for quality control. For fungi, demultiplexed sequence data were trimmed with q2-ITSxpress (Rivers et al., 2018) and an unmerged ITS2 sequence from a fungal amplicon sequencing dataset was extracted for quality control. DADA2 (Callahan et al., 2016) was used to filter, trim, denoise, and merge the data. Chimeric sequences were removed using the consensus method. For phylogenetic diversity analysis, all observed amplicon sequence variants (Callahan et al., 2017), that is, 100% operational taxonomic units (OTUs), were aligned using the MAFFT program plugin via q2-alignment (Katoh et al., 2002) to construct a phylogenetic tree with FastTree2 via q2-phylogeny (Price et al., 2010). Taxonomic classification was assigned using the SILVA database (version 132), specific for the V3-4 region of 16S rRNA genes, and the UNITE fungal ITS database (version 8.2), specific to the ITS2 region of fungal rRNA genes (Nilsson et al., 2019). All taxonomic classifications were implemented using QIIME2 and assigned using the naïve Bayesian algorithm. The alpha diversity (observed OTUs and Faith's phylogenetic diversity) was estimated using q2-diversity at the OTU level.

Statistical analysis

Data for fermentation products, bacterial and fungal copy numbers, and the alpha diversity of bacterial and fungal microbiota were subjected to the non-parametric Kruskal-Wallis sum-rank test, and the Dunn-Bonferroni pairwise comparison for post hoc test was used to determine significant differences between two groups at a probability of <0.05. These analyses were performed using IBM SPSS Statistics (Version 26.0, IBM Co., Armonk, NY, USA, 2019)

The beta-diversity of bacterial and fungal microbiota was calculated based on the Bray-Curtis distance, using the filtered genus-level taxa and was visualized using principal coordinate analysis (PCoA) with Primer-E software (version 7, Quest Research Ltd., Auckland, New Zealand). Discriminant vectors with a Pearson correlation >0.7 were considered significant. Correlations between fermentation products and the relative abundance of bacterial and fungal genera were calculated using Spearman's rank correlation

coefficient. Significant correlations at a probability of <0.05 between the fermentation products and dominant genera were visualized by network analysis using the Cytoscape software (version 3.8.2) (Shannon et al., 2003).

4.2. Results

Fermentation products

The DM contents of silages didn't show a difference (P>0.05) between bunker silos, whereas the pH value and fermentation products differed (P<0.05) between bunker silos. Bunker A, C and E showed high pH value, more acetic acid and 1-propanol contents, while less lactic acid and ethanol contents; whereas bunker B and D showed low pH value, more lactic acid and ethanol contents, while less acetic acid content. The 1-propanol was less or not detectable in bunker B and D. The ratio of lactic acid to acetic acid content was low in bunker B and D than A, C and E. The ammonia nitrogen didn't show a difference between bunker silos (Table 4.1).

A large amount (more than 12.0 g/kg DM) of 1,2-propanediol was detected in several samples of bunker C, D and E, while a small amount (2.97-4.34 g/kg DM) of which was detected in bunker B, and it was not detected in bunker A. The propionic acid was frequently detected in bunker C and E and exceeded 5 g/kg DM in several samples, and was detected at 4.69 and 4.94 g/kg in two samples of bunker D, but it was not detected in bunker A and B. The marginal content of butyric acid was detected in bunkers B, C, D and E, and it was also not detected in bunker A. One sample of bunker E showed a high pH value (4.41) and very low levels of lactic and acetic acid contents (Table 4.2).

Table 4.1 Dry matter, pH value and fermentation products of five bunker silos.

		A	B	C	D	E	Kruskal-Wallis test
DM	g/kg	239±16.3	257±52.8	273±16.2	230±26.7	265±38.2	0.059
pH		3.96±0.13a	3.55±0.10b	3.85±0.08a	3.63±0.09b	3.95±0.23a	<0.01
Lactic acid	g/kg DM	59.7±15.4ab	86.7±35.7a	42.9±11.2b	72.8±24.9ab	47.3±26.0ab	<0.05
Acetic acid	g/kg DM	56.6±9.14a	29.7±15.9b	64.3±18.2a	33.1±7.85b	45.6±26.5ab	<0.01
L/A		1.07±0.28b	3.70±1.78a	0.70±0.21b	2.31±1.12a	0.99±0.31b	<0.01
Ethanol	g/kg DM	12.5±1.93b	34.4±14.5ab	7.82±2.09c	32.2±10.9a	10.6±4.70bc	<0.01
1-Propanol	g/kg DM	16.9±1.95a	0.58±1.42b	13.2±3.55a	2.11±3.26b	13.3±8.49a	<0.01
1,2-Propanediol	g/kg DM	ND	3.02±1.54	7.00±10.9	6.85±6.46	2.51±6.15	-
Butyric acid	g/kg DM	ND	0.41±1.01	0.37±0.91	0.75±1.18	0.08±0.19	-
Propionic acid	g/kg DM	ND	ND	6.73±6.33	1.60±2.49	7.47±5.73	-
Ammonia-N	g/kg DM	1.03±0.17	0.87±0.18	1.10±0.28	0.73±0.21	0.82±0.43	0.135

DM, dry matter; L/A, lactic to acetic acid ratio; Values in the same row with different following letters (a–c) are significantly different.

Table 4.2 Dry matter, pH value and fermentation products of all collected samples in five bunker silos.

	Dry matter	pH	Lactic acid	Acetic acid	L/A	Ethanol	1-Propanol	1,2-Propanediol	Butyric acid	Propionic acid	Ammonia-N
	g/kg		g/kg DM	g/kg DM		g/kg DM	g/kg DM	g/kg DM	g/kg DM	g/kg DM	g/kg DM
A-1	254	3.85	64.7	51.2	1.26	13.08	16.79	ND	ND	ND	0.81
A-2	231	4.00	52.7	51.3	1.03	10.31	16.12	0.00	ND	ND	1.22
A-3	251	3.87	68.8	49.2	1.40	12.59	16.88	0.00	ND	ND	0.93

A-4	253	3.89	52.9	53.0	1.00	11.79	14.49	0.00	ND	ND	0.96
A-5	213	3.92	81.5	72.7	1.12	15.95	20.41	0.00	ND	ND	1.01
A-6	231	4.21	37.3	62.4	0.60	11.45	16.41	0.00	ND	ND	1.23
B-1	254	3.54	118.6	27.3	4.34	52.18	ND	4.34	ND	ND	0.93
B-2	282	3.55	104.1	24.4	4.27	43.54	ND	3.42	ND	ND	0.99
B-3	171	3.73	17.7	61.2	0.29	9.55	ND	ND	2.47	ND	0.53
B-4	242	3.45	103.3	25.2	4.10	36.61	ND	3.67	ND	ND	0.99
B-5	332	3.50	90.4	16.4	5.50	29.09	ND	2.97	ND	ND	0.82
B-6	263	3.52	86.1	23.4	3.68	35.52	3.48	3.70	ND	ND	0.97
C-1	251	3.86	42.2	76.3	0.55	7.37	13.22	20.71	ND	ND	1.28
C-2	281	3.98	32.3	58.4	0.55	5.94	6.91	21.30	ND	ND	1.30
C-3	256	3.87	43.3	93.4	0.46	7.67	14.36	ND	ND	16.83	1.29
C-4	274	3.73	36.8	45.4	0.81	7.21	13.61	ND	2.24	7.52	0.61
C-5	291	3.84	38.5	47.7	0.81	6.79	13.44	ND	ND	6.58	0.91
C-6	286	3.83	64.4	64.6	1.00	11.90	17.89	ND	ND	9.42	1.18
D-1	230	3.72	91.3	34.9	2.62	40.18	ND	8.55	ND	ND	0.87
D-2	230	3.69	75.4	37.9	1.99	36.19	ND	15.25	ND	ND	0.83
D-3	232	3.67	83.9	44.9	1.87	38.09	ND	12.83	ND	ND	0.89
D-4	204	3.66	36.2	29.3	1.24	16.64	6.53	ND	1.81	4.69	0.34
D-5	278	3.53	100.3	22.7	4.41	41.77	ND	4.48	ND	ND	0.82
D-6	206	3.52	49.6	28.6	1.74	20.26	6.10	ND	2.66	4.94	0.66
E-1	270	3.87	63.1	46.8	1.35	11.39	11.68	ND	ND	11.13	0.98
E-2	300	3.83	41.3	45.9	0.90	10.46	15.64	ND	ND	7.55	0.85
E-3	305	3.77	41.3	31.5	1.31	9.58	8.62	15.07	ND	ND	0.63
E-4	201	4.41	1.77	3.28	0.54	2.26	ND	ND	0.47	0.98	0.09
E-5	267	3.91	75.9	70.7	1.07	14.71	21.09	ND	ND	11.89	1.04
E-6	247	3.93	60.3	75.3	0.80	15.34	22.94	ND	ND	13.26	1.34

DM, dry matter; L/A, lactic to acetic acid ratio.

Microbial rRNA copy numbers

The copy number of bacteria was higher than fungi in most silage samples from all bunkers. There was no clear difference in bacterial copy number between bunkers. Bunker B and D showed a high level of fungal copy number, and the distinctly high level (>10^5 copies/g silage) of fungal copy number was observed in one sample of bunker B and E and three samples of bunker D (Table 4.3).

Table 4.3 Bacterial and fungal copy numbers, α diversities of the bacterial and fungal community of five bunker silos.

			A	B	C	D	E	Kruskal-Wallis test
	Total bacteria	log copies/g silage	7.76±0.16	6.91±0.93	7.58±0.33	7.09±0.98	7.11±1.06	0.315
Bacteria	Observed OTUs		33.3±8.73b	104±101ab	35.0±11.0ab	66.8±71.9ab	121±38.6a	<0.05
	Faith PD		5.88±0.75ab	10.3±6.60a	4.39±0.77b	6.98±4.27ab	10.4±2.57a	<0.01
	Total fungi	log copies/g silage	3.44±0.26b	4.32±1.32ab	3.30±0.23b	5.22±1.66a	3.00±1.64b	<0.01
Fungi	Observed OTUs		27.7±3.39b	30.8±7.36b	26.5±2.74b	27.2±4.71b	58.3±22.5a	<0.01
	Faith PD		9.60±1.11b	10.4±1.51b	9.45±1.02b	9.64±1.10b	15.5±6.79a	<0.01

OTUs, operational taxonomic units; PD, phylogenetic diversity; Values in the same column with different following letters (a–d) are significantly different.

Table 4.4 Bacterial and fungal copy numbers, α diversities of the bacterial and fungal community of all collected samples in five bunker silos.

	Bacteria				Fungi			
	Total bacteria	Filtered sequence number	Observed OTUs	Faith PD	Total fungi	Filtered sequence number	Observed OTUs	Faith PD
	log copies/g silage				log copies/g silage			
A-1	7.85	43209	25	5.20	3.49	11415	26	9.28
A-2	7.56	54673	37	5.14	3.52	12682	31	10.70
A-3	7.86	40271	27	6.28	3.29	13389	31	10.93
A-4	7.99	42798	42	6.95	3.48	11373	24	8.38
A-5	7.67	51020	25	5.35	3.03	9847	30	9.93
A-6	7.64	58538	44	6.39	3.83	11881	24	8.37
B-1	7.16	30403	41	8.06	3.78	16286	37	11.73
B-2	6.90	16429	24	5.81	6.92	9817	18	7.77
B-3	8.44	44219	46	5.03	4.42	12646	34	11.94
B-4	6.26	90337	154	13.25	3.45	15190	35	10.44
B-5	5.69	78155	288	22.44	3.79	14163	35	10.50
B-6	7.02	52797	69	7.29	3.55	9449	26	9.88
C-1	7.39	56537	27	4.11	2.89	11442	23	8.76
C-2	7.94	46450	21	3.70	3.45	10773	24	9.10
C-3	7.08	42824	42	5.18	3.42	12475	29	10.27
C-4	7.57	29063	52	5.54	3.36	13200	27	9.23
C-5	7.54	43286	36	3.95	3.18	11128	26	8.29
C-6	7.94	43113	32	3.85	3.51	11476	30	11.03
D-1	5.80	60297	206	15.16	5.83	6960	22	8.11
D-2	6.00	942	13	3.35	8.08	11035	23	8.86
D-3	7.24	48698	34	5.41	5.66	10931	25	9.49
D-4	8.25	69764	70	6.88	3.76	10683	31	10.46
D-5	7.69	60462	17	4.01	4.01	10958	34	11.19
D-6	7.56	60556	61	7.07	4.00	10157	28	9.73
E-1	6.03	88160	173	12.11	0.62	11595	59	13.53
E-2	6.68	42398	135	12.38	2.64	8707	53	13.22
E-3	5.93	44157	127	10.46	2.58	7300	39	11.71
E-4	8.47	31477	131	12.39	5.56	10039	102	29.30
E-5	7.53	45289	103	9.35	2.66	15900	45	11.99
E-6	8.01	36296	57	5.81	3.92	10006	52	13.46

OTUs, operational taxonomic units; PD, phylogenetic diversity.

Bacterial microbiota

A total of 1,452,618 filtered 16S rRNA high-quality sequences were obtained to classify 1,079 OTUs from 30 samples. The bunker B and D showed similarly high levels of observed OTUs and the Faith PD, while the bunker A, C and D showed similarly low levels of these two diversity indices. These two

indices were extremely high in two samples of bunker B, one sample of bunker D, and five samples of bunker E.

The abundance of *Lactobacillus* was more than 50% in most silage samples of all bunkers. The abundant genus of *Acetobacter* was observed in all silage samples of bunker B and one sample of bunker E, and the abundance of it was more than 50% in one sample of bunker B and C, separately. The abundant genus of *Aeriscardovia* was observed in five silage samples of bunker A, three samples of bunker C and three samples of bunker E, with an abundance of more than 10% in most of these samples. The abundant genus of *Paenibacillus* was observed in bunker B, with an abundance of more than 15% in one sample. The abundant genera of unclassified Muribaculaceae, unclassified Lachnospiraceae, *Eubacterium* coprostanoligenes group, Clostridia UCG-014, *Bacteroides* and *Alistipes* were observed in bunker E, and more than 10% abundance of *Pseudomonas* was also observed in one sample of bunker E (Figure 4.2).

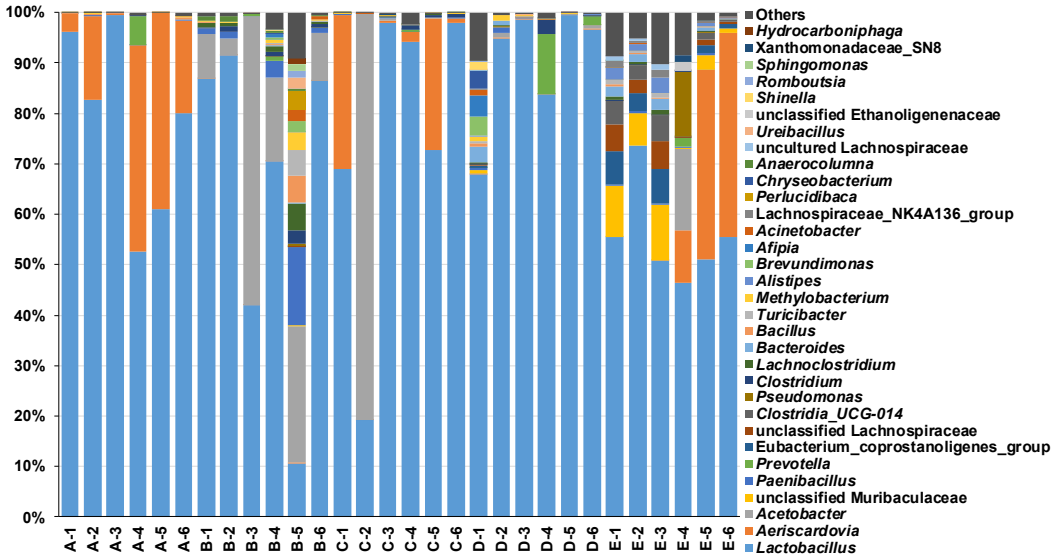

Figure 4.2 Relative abundances of 32 major genera (>1% in abundance) of the fungal microbiota of bunker silos. A, B, C, D and E indicate five bunker silos, respectively. The numbers from one to six indicate the sample locations collected from each silo.

Based on the PCoA plots (Figure 4.3), most samples formed the biggest group, and three samples of bunker E formed a separate group. These two groups were characterized by *Lactobacillus* genus. Four samples of bunker A, two samples of bunker C and two samples of bunker E formed a separated group, which characterized by genera of *Lactobacillus* and *Aeriscardovia*. Four samples (two samples of bunker B, one sample of C and E) were separately characterized by *Acetobacter* genus.

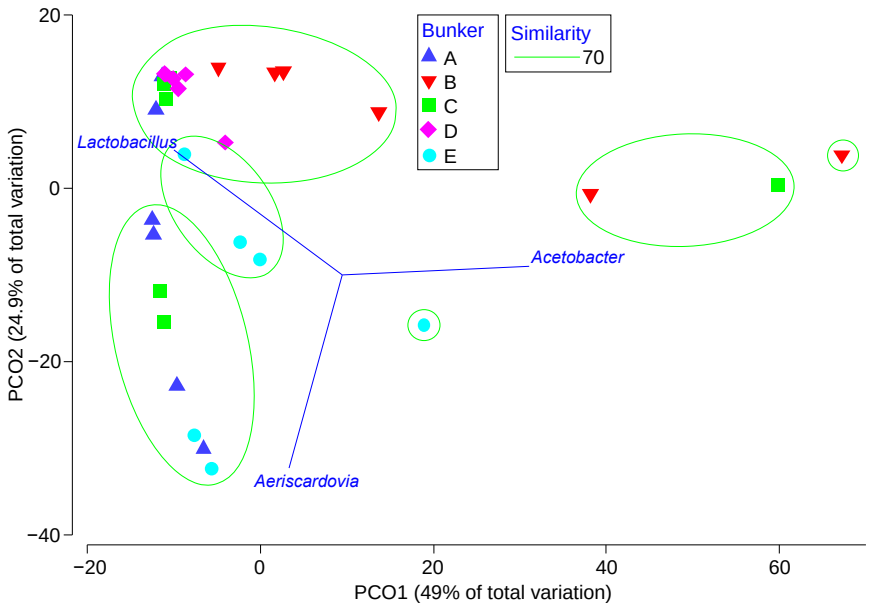

Figure 4.3 Beta diversity of the bacterial microbiota of bunker silos. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together on the ordination have a more similar microbiota. The operational taxonomy unit with a Pearson's correlation of >0.7 is overlaid on the plot as vectors. Green circles denote samples enclosed in the same group at a 70% similarity level. A, B, C, D and E indicate five bunker silos, respectively.

Fungal microbiota

A total of 342,903 filtered ITS2 high-quality sequences were obtained to classify 381 OTUs. The observed OTUs and the Faith PD were significantly higher in bunker E than A, B, C and D, and there was no difference for these two indices between bunker A, B, C and D. These two indices were extremely high especially in one sample of bunker E.

The abundance of *Candida* was more than 50% in most silage samples of all bunkers. The abundant genus of *Monascus* was observed in three samples of bunker D and one sample of bunker B, and it was the most abundant fungal genus in one sample of bunker B and D, separately. The *Kazachstania* was the most abundant fungal genus in one sample of bunker D. Five samples of bunker E showed obviously high abundant *Issatchenkia*, *Kazachstania* and *Nakaseomyces*, and the *Vishniacozyma* was the most abundant fungal genus in another sample of bunker E (Figure 4.4).

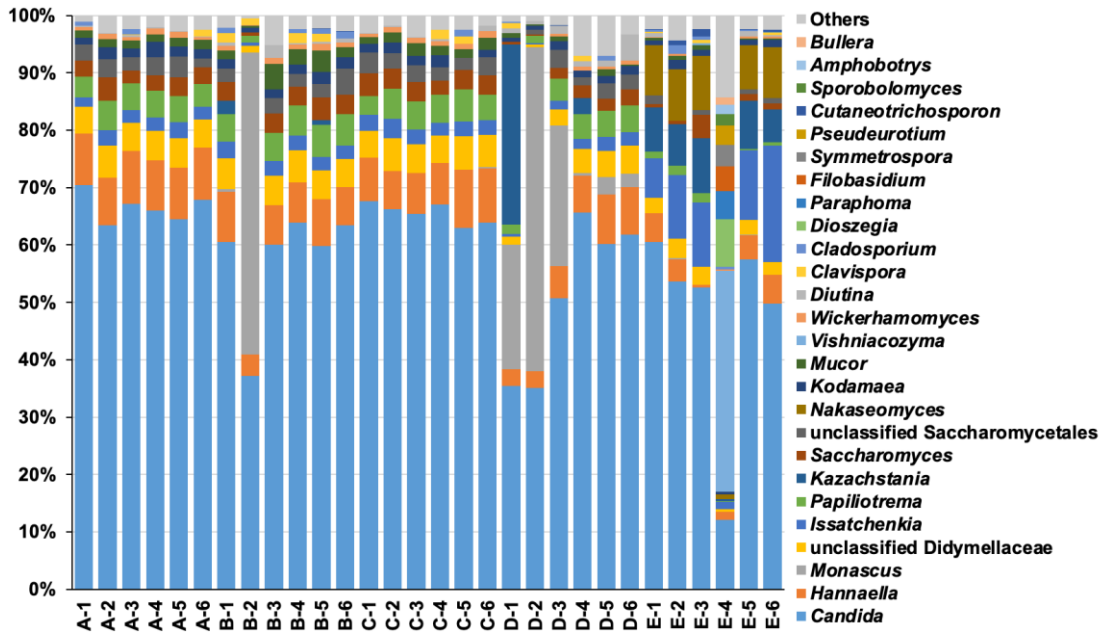

Figure 4.4 Relative abundances of 26 major genera (>1% in abundance) of the fungal microbiota of bunker silos. A, B, C, D and E indicate five bunker silos, respectively. The numbers from one to six indicate the sample locations collected from each silo.

Based on the PCoA plots (Figure 4.5), most samples formed the biggest group, and five samples of bunker E formed a separate group. These two groups were characterized by genera of *Candida*, *Hannaella*, unclassified Didymellaceae, *Papiliotrema*, unclassified Saccharomycetales, *Saccharomyces* and *Wickerhamomyces*. Three samples (two samples of bunker D and one sample of B) were separately characterized by *Monascus* genus. One sample of bunker E was separately characterized by genera of *Vishniacozyma*, *Dioszegia*, *Paraphoma*, *Filobasidium*, *Symmetrospora*, *Pseudeurotium*, *Sporobolomyces*, *Amphobotrys* and *Bullera*.

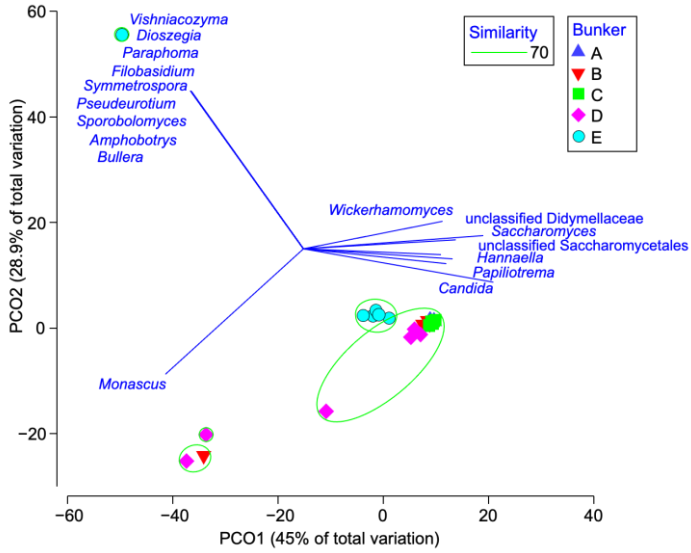

Figure 4.5 Beta diversity of the fungal microbiota of bunker silos. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together on the ordination have a more similar microbiota. The operational taxonomy unit with a Pearson's correlation of >0.7 is overlaid on the plot as vectors. Green circles

denote samples enclosed in the same group at a 70% similarity level. A, B, C, D and E indicate five bunker silos, respectively.

Relationships between the fermentation products and core genera of bacteria and fungi

A correlation network indicated that the lactic acid was positively related to ethanol and 1,2-propanediol, while the acetic acid was positively related to 1-propanol and ammonia. The fermentation products of lactic acid and ethanol were uniformly positively related to the abundance of *Monascus* genus. The lactic acid was also positively related to *Paenibacillus*. The acetic acid and 1-propanol were uniformly positively related to *Aeriscardovia*, *Candida* and *Issatchenkia*, and negatively related to *Acetobacter*. The 1,2-propanediol was positively related to the abundance of *Acetobacter* genus, but negatively related to *Aeriscardovia*. The abundance of *Lactobacillus* was unrelated to any fermentation product, but positively related to the fungal genus of *Monascus* (Figure 4.6).

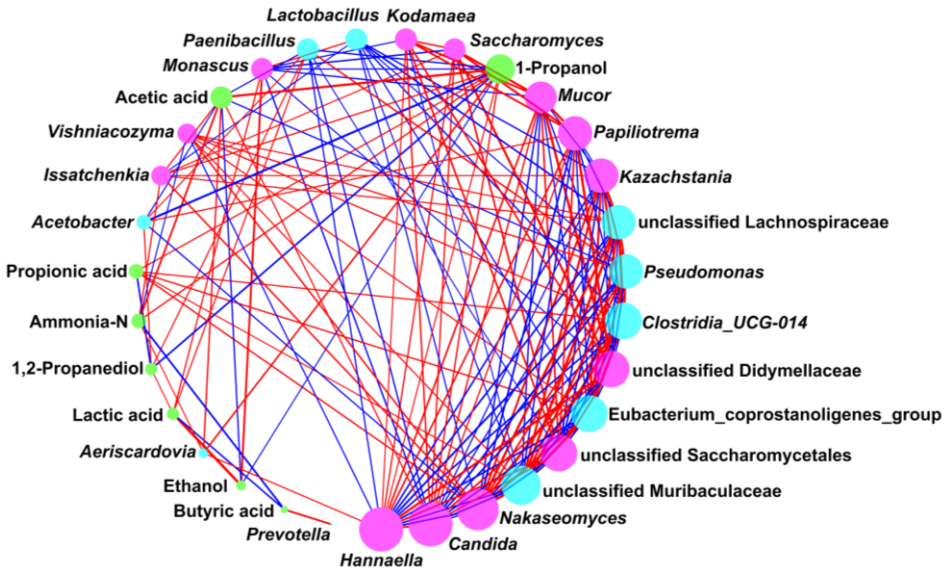

Figure 4.6 Correlation network diagram for fermentation products and major bacterial and fungal genera of bunker silos. Green, blue, and purple nodes represent the fermentation product, bacterial genus, and fungal genus, respectively. The node size represents the degree of centrality, which is calculated as the number of edges connecting a node to others. The Spearman's Rho of two nodes is indicated by the edge width, with red and blue edges describing the positive and negative relationships between the two nodes.

4.3. Discussion

From the major fermentation products of lactic acid, acetic acid, ethanol and 1-propanol, the fermentation patterns were divided into the lactic acid (17.7-118 g/kg DM) and ethanol (9.55-52.2 g/kg DM) dominant type (bunker B and D), and the acetic acid (3.28-93.4 g/kg DM) and 1-propanol (0-22.9 g/kg DM) dominant type (bunker A, C and E). Silage management factors by farmers, such as delayed sealing and air infiltration during the fermentation process, should be the reason of such contrary fermentation pattern of corn silage, because these five farms were at same area and arranged for the same contractor. The large variation of these fermentation products within a bunker was also observed in our study corresponding to previous bunker silo surveys (Y Li & Nishino, 2011; Tabacco et al., 2011)(Weiss et al., 2016). The crops for ensiling could be harvested at inappropriate stages because the DM content was also variable within a bunker, and it may be caused by inclement weather, equipment trouble or scheduling problems with a contractor. Although the lactic acid content is generally different duo to

nonuniform distribution of DM or different air penetration in the different sampling location within a bunker silo (L. D'Amours & P. Savoie, 2005), such as the top and bottom layers, near the wall and centre, lateral and perpendicular differences for lactic acid and acetic acid were not observed in this study, which supports previous findings that similar high concentrations of lactic acid and the low pH observed in silage samples taken from the center and walls (Kraut-Cohen et al., 2016), and neither the lateral nor perpendicular difference was repeated across the two years of bunkers (Y Li & Nishino, 2011).

Bacterial and fungal communities didn't show a clear difference between these two fermentation types. The bacterial communities were found to be highly variable between and within bunkers.

The *Lactobacillus* was the most abundant bacterial genus, which was not related with any fermentation product but positively correlated with a fungal genus of *Monascus* based on the network analysis. This indicates that even if the bacterial population of silage predominated by *Lactobacillus*, it cannot be said that silage could be stored stably.

The 1-propanol was detected frequently and at high levels (more than 10 g/kg DM) in bunkers A, C and E. The 1-propanol can be formed by *Lactobacillus diolivorans* in conjunction with propionic acid via conversion of 1,2-propanediol (Krooneman et al., 2002). The positive relationship between 1-propanol and propionic acid, and the negative relationships between 1,2-propanediol and 1-propanol and propionic acid were demonstrated by network analysis; thus, the 1-propanol could be produced from the metabolism of 1,2-propanediol in corn silage of bunker, which corresponds to previous findings (Weiss et al., 2016; Hafner et al., 2014; Y Li & Nishino, 2011; Kristensen et al., 2010). However, no relationship between 1-propanol and *Lactobacillus* could be demonstrated in this study, and it is difficult to determine which species of *Lactobacillus* account for this high product of 1-propanol because of lacking identification of species-level by NGS.

The surprisingly high abundant *Acetobacter* was detected in bunkers B, C and E. The *Acetobacter* can oxidize ethanol to produce acetic acid under aerobic conditions (Kumiko et al., 2001), but much high acetic acid was not observed in these samples. Although the presence of acetic acid bacteria in silage is considered undesirable because it can initiate aerobic deterioration and degrade lactic acid and acetic acid to produce carbon dioxide and water (Spoelstra et al., 1988; Drouin & Ferrero, 2020), there was no correlation between *Acetobacter* with lactic and acetic acid, and aerobic mold of *Monascus*. Interestingly, the 1,2-propanediol was positively correlated with *Acetobacter* and *Paenibacillus* based on network analysis. Several samples showed a large amount (more than 12.0 g/kg DM) of 1,2-propanediol that could be produced from the anaerobic metabolism of lactic acid by *Lactobacillus* sp. in silage fermentation (Oude Elferink et al., 2001). The *Acetobacter pasteurianus* was also detected by semi-quantitative denaturing gradient gel electrophoresis analysis in bunker silo (Y Li & Nishino, 2011). The abundant genus of *Paenibacillus*, as facultative anaerobic organisms, was also observed in bunker B that showed high level of lactic acid and abundant *Acetobacter*, and it was positively related with lactic acid and *Acetobacter* by network analysis. The appearance of abundant *Acetobacter* and *Paenibacillus* might be caused by more air infiltration into the silo face of bunker B. The *Paenibacillus polymyxa* can survive in the late phase of ensiling fermentation and appeared incapable of fermenting lactate and producing butyrate (Driehuis et al., 2016). It was also reported that the xylanase-producing *Paenibacillus panacisoli* could enhance the growth of lactic acid bacteria in corn stover silage (Xu et al., 2018). Although which species of *Paenibacillus* appeared in this study was limited by NGS, there was no sign of aerobic spoilage

from the pH value and fermentative products in bunker B and none of the farmers acknowledged problems with the silage we sampled. Therefore, the presence of *Acetobacter* spp. and *Paenibacillus* spp. might not be an indication of aerobic instability after silo opening, and it remains to determine how they could grow and survive in the silage environment.

Interestingly, the abundant *Aeriscardovia* was also observed as a non-conventional bacterium in Bunker A, C and E. The genus *Aeriscardovia* belongs to the family Bifidobacteriaceae, but it can grow in air (Simpson et al., 2004). From the fermentation products, high level of acetic acid content and low level of lactic acid content may be associated with *Aeriscardovia* in bunker A, C and E, because Bifidobacteriaceae can metabolize glucose to produce generally about three molecules of acetic acid and two molecules of lactic acid per two moles of glucose (Mattarelli & Sgorbati, 2018). The bacterial genera of unclassified Muribaculaceae, unclassified Lachnospiraceae, Eubacterium_coprostanoligenes_group, Clostridia UCG-014, *Bacteroides* and *Alistipes*, as members of gastrointestinal tract microbiota, were prevalent in Bunker E, but these bacteria were rarely found in other bunkers.

A sample from bunker B, and three samples from bunker D showed a noticeably high abundance of *Monascus purpureus*. Moreover, the fungal copy numbers of these samples were obviously high compared with other samples. *Monascus purpureus* was found as the dominant fungi during the late ensiling of air-stressing uninoculated corn silages (Kung et al., 2021). Molds from the genus *Monascus* are common in poorly managed corn silages and undesirable especially because those fungi can produce mycotoxins (Jonsson, 1991; Cheli et al., 2013; Schneweis et al., 2001). Therefore, the high copy numbers of fungi and high abundant *Monascus* in silages from silo high probably indicated that the silage is undergoing an advanced stage of aerobic deterioration even the fermentation product did not show a clear difference.

The abundant *Monascus* was positively correlated with fermentation products of lactic acid and ethanol, but negatively correlated with fermentation product of 1-propanol, fungal genera of *Candida*, *Saccharomyces*, *Kodamaea*, unclassified Didymellaceae, *Wickerhamomyces* and *Mucor* based on network analysis. The main yeast genera were *Candida, Issatchenkia, Kazachstania*, *Nakaseomyces* and *Saccharomyces*, which was not correlated with fermentation products of lactic acid and ethanol based on network analysis. The most abundant fungal genus was *Candida*, which was positively correlated with acetic acid and 1-propanol. *Candida intermedia* was clearly found in *L. buchneri*-inoculated corn silage, but undetectable after exposure to air by semi-quantitative denaturing gradient gel electrophoresis analysis (Yanbing Li & Nishino, 2011). In addition, the *Candida* was the prevalent fungal genus associated with terminal silage of corn, barley, oat or sugarcane (C. Bai et al., 2021; Duniere et al., 2017; Keshri et al., 2018; B. Liu et al., 2019; Wang et al., 2020). Therefore, the silage present a high abundant *Candida* is highly suggestive of stably stored ensiling, although it was reported that the *Candida*, as lactic acid-assimilating yeasts, was associated with aerobic deterioration of silages (Jiang et al., 2020). The fungal genus of *Issatchenkia* was also positively correlated with acetic acid and 1-propanol, thus, the new yeast inoculant of these two fungi need to address the potential for improving the aerobic stability of silage.

4.4. Summary

The fermentation patterns were divided into two types: lactic acid and ethanol (B and D) and acetic acid and 1-propanol (A, C, and E) as the prevalent products. *Lactobacillus* was the predominant bacterial genus in all farms, while some silage samples showed high abundances of *Acetobacter* and *Aeriscardovia*. The fungal microbiota was dominated by *Candida* regardless of the farms and sampling locations. Network analysis indicated that the *Lactobacillus* abundance was not related to any fermentation product contents, whereas positively related to the *Monascus* abundance. *Aeriscardovia*, which belongs to Bifidobacteriaceae and may produce more acetic acid than lactic acid, can be considered a new inoculant. Unlike the findings in the guinea grass silage, the integrated bacterial and fungal microbiota data did not greatly improve the understanding of whole crop corn silage fermentation

CHAPTER 5 Conclusions

Firstly, direct-cut (DC) and wilted (WT) guinea grass silages were prepared to examine the mechanisms and control procedures of acetic acid fermentation. The silos were stored at moderate (25℃) and high (40℃) temperatures. Lactic acid was the primary fermentation product during the initial ensiling. After two months, the lactic acid content was reduced to a negligible level, and large amounts of acetic acid, butyric acid, and ethanol were produced in DC silage stored at 25℃. The lactic acid reduction and acetic acid increase were suppressed in DC silage stored at 40℃. The PCoA and network analyses showed that *Lactobacillus*, unclassified Enterobacteriaceae, *Wallemia*, *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* could be involved in acetic acid fermentation. *Lactobacillus* and *Wallemia* were identified as promoting factors and unclassified Enterobacteriaceae, *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* as suppressing factors.

Secondly, guinea grass silage that exhibited lactic acid-rich fermentation was examined. Molasses were used to facilitate lactic acid production and silos were stored at 25℃ and 40℃. The acetic acid content did not exceed the lactic acid even after 2 months in all silages. Regardless of the storage temperature and molasses addition, the unclassified Enterobacteriaceae, *Lactobacillus*, *Lactococcus*, *Enterococcus,* and *Pediococcus* were prevalent bacteria. *Wallemia*, *Saitozyma*, and *Ganoderma* were prevalent fungi in DC and WT silages. Network analysis indicated that the lactic acid content was positively related to the *Pediococcus, Weissella,* and *Wallemia* abundances, and negatively related to the unclassified Enterobacteriaceae, *Acinetobacter*, *Ochrobactrum*, *Enterococcus*, *Cladosporium, Acremonium, Saitozyma,* and *Moesziomyces* abundances. The lactic acid-rich fermentation of guinea grass silage could be obtained by the sustained activity of *Pediococcus*, *Lactococcus*, and *Enterococcus* rather than the suppression of *Lactobacillus*.

Lastly, a practical survey of large-scale silage management was performed. Five dairy farms (A, B, C, D, and E) producing whole crop corn silage using a bunker silo were visited. Silage samples were taken from 6 locations in the upper and lower layers and near the side-wall and the center. The fermentation patterns were divided into two types: lactic acid and ethanol (B and D) and acetic acid and 1-propanol (A, C, and E) as the prevalent products. *Lactobacillus* was the predominant bacterial genus in all farms, while some silage samples showed high abundances of *Acetobacter* and *Aeriscardovia*. The fungal microbiota was dominated by *Candida* regardless of the farms and sampling locations. Network analysis indicated that the *Lactobacillus* abundance was not related to any fermentation product contents, whereas positively related to the *Monascus* abundance. *Aeriscardovia*, which belongs to Bifidobacteriaceae and may produce more acetic acid than lactic acid, can be considered a new inoculant. Unlike the findings in the guinea grass silage, the integrated bacterial and fungal microbiota data did not greatly improve the understanding of whole crop corn silage fermentation.

Abstract

Microbiota analysis using DNA information has become more sophisticated and easily accessible in the past 20 years. Many studies have been conducted to elucidate the microbiota involved in fermentation and aerobic deterioration of silage. However, many studies have confirmed the well-established knowledge that the rapid growth of lactic acid bacteria after sealing determines the success or failure of silage fermentation. The fact that most microbiota analyses have focused on bacteria may be the reason.

In this dissertation, three experiments were carried out to obtain integrated information on the bacterial and fungal microbiota involved in the anaerobic storage of forages. The first two studies examined guinea grass silage prepared with laboratory-scale silos and the third examined whole-crop corn silage produced in a large-scale bunker silo. The acetic acid fermentation found in tropical grass silage has been a topic for which the mechanisms and control procedures have not been clarified. Likewise, there is a continuous demand to improve technology and management of large-scale forage conservation.

1. Microbiota of guinea grass silage exhibiting intensive acetic acid fermentation

Direct-cut (DC, 170 g dry matter [DM]/kg) and wilted (WT, 323 g DM/kg) guinea grass were stored in a laboratory silo at moderate (25℃) and high (40℃) temperatures. Bacterial and fungal microbiota were assessed at 3 days, 1 month, and 2 months after ensiling. Lactic acid was the primary fermentation product during the initial ensiling period, and a high *Lactococcus* abundance was found in DC silage. After two months, the lactic acid content was reduced to a negligible level, and large amounts of acetic acid, butyric acid, and ethanol were found in DC silage stored at 25℃. The lactic acid reduction and acetic acid increase were suppressed in DC silage stored at 40℃. Increased abundances of *Lactobacillus*, *Clostridium*, and *Wallemia*, and decreased abundance of *Saitozyma*, *Papiliotrema*, and *Sporobolomyces* were observed in DC silages from day 3 to the end of the 2 months period. Wilting suppressed acid production, and lactic and acetic acids were found at similar levels in WT silages, regardless of the temperature and storage period. The abundance of *Lactobacillus* was lower in WT than in DC silages. The unclassified Enterobacteriaceae was the most prevalent bacteria in DC (38.1–64.9%) and WT (50.9–76.3%) silages, and the abundance was negatively related to the acetic acid content. The PCoA and network analyses showed that *Lactobacillus*, unclassified Enterobacteriaceae, *Wallemia*, *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* could be involved in acetic acid fermentation. *Lactobacillus* and *Wallemia* were identified as promoting factors and unclassified Enterobacteriaceae, *Saitozyma*, *Sporobolomyces*, and *Papiliotrema* as suppressing factors. Regardless, the abundance of *Lactobacillus* was most distinctively related to changes in the acetic acid content during fermentation. Storage at 40℃ suppressed acetic acid fermentation in DC and WT silage, indicating that high storage temperatures may not adversely affect silage fermentation. Mixed fermentation and 2,3-butanediol fermentation were not found, even with a high abundance (>60%) of unclassified Enterobacteriaceae.

2. Microbiota of guinea grass silage exhibiting lactic acid fermentation

Direct-cut (DC, 176 g DM/kg) and wilted (WT, 266 g DM/kg) guinea grass silage was prepared in a laboratory-scale silo with and without molasses (10 g/kg wet weight). The silo was stored at moderate (25℃) and high (40℃) temperatures for 2 weeks and 2 months. Lactic acid dominated the fermentation in DC and WT silages regardless of the molasses addition and storage temperature. Although the lactic

to acetic acid ratio was kept >1.0 throughout the ensiling, the acetic acid production was enhanced when the storage was extended to 2 months. The molasses addition increased the lactic acid and decreased the acetic acid in DC silage stored at 25℃; however, the effect was not observed and molasses enhanced the acetic acid production in DC silage stored at 40℃. The lactic and acetic acid contents were decreased by molasses in WT silage stored at 25℃, whereas molasses increased the acid production in WT silage stored at 40℃. The bacterial and fungal populations were decreased when stored at 40℃ compared to 25℃. Regardless of the storage temperature and molasses addition. Unclassified Enterobacteriaceae, *Lactobacillus*, *Lactococcus*, *Enterococcus,* and *Pediococcus* were prevalent bacteria in DC and WT silages. When stored at 25℃ for 2 months, *Lactobacillus* was the most prevalent in DC and WT silage regardless of the molasses addition. In contrast, *Pediococcus* was the most abundant in DC silage stored at 40℃, and unclassified Enterobacteriaceae and *Lactobacillus* were the two most abundant in WT silage stored at 40℃. *Wallemia*, *Saitozyma*, and *Ganoderma* were prevalent fungi in DC and WT silages, whereas *Candida*, *Diutina*, *Acremonium*, and *Myrmecridium* were detected at the varying abundance in several samples. *Wallemia* showed a higher abundance in DC silage with high levels of lactic and acetic acids. In contrast, *Saitozyma* showed a higher abundance in WT silage with low levels of lactic and acetic acids. Network analysis indicated that the lactic acid content was positively related to the *Pediococcus, Weissella,* and *Wallemia* abundances, and negatively related to the unclassified Enterobacteriaceae, *Acinetobacter*, *Ochrobactrum*, *Enterococcus*, *Cladosporium, Acremonium, Saitozyma,* and *Moesziomyces* abundances. The lactic acid fermentation of guinea grass silage could be obtained by the sustained activity of *Pediococcus*, *Lactococcus*, and *Enterococcus* rather than the suppression of *Lactobacillus*.

3. Microbiota of whole crop corn silage produced in a bunker silo

A practical survey of large-scale silage management was performed. Five dairy farms (hereafter referred to as bunkers A, B, C, D, and E) producing whole crop corn silage using a bunker silo were visited in November 2020 and January 2021. All farms used the same harvester and the *Lactobacillus buchneri* inoculant. Silage samples were taken from 6 locations in the upper and lower layers and near the side-wall and the center. The storage period varied from 3 to 15 months. The fermentation patterns were not affected by the sampling location but were divided into two types; lactic acid and ethanol (B and D) and acetic acid and 1-propanol (A, C, and E) as the prevalent products. The types of fermentation appeared unrelated to the ensiling period. Although no clear differences were seen for the bacterial population, the lactic acid-ethanol silage had higher fungal populations. *Lactobacillus* was the predominant bacterial genus in all bunkers, while some silage samples showed high abundances of *Acetobacter* (> 50%) and *Aeriscardovia* (30-40%). The fungal microbiota was dominated by *Candida* regardless of the bunkers and sampling locations, whereas high abundances of *Monascus* and *Kazachstania* were observed in limited samples. Network analysis indicated that the *Lactobacillus* abundance was not related to any fermentation products, whereas positively related to the *Monascus* abundance. Likewise, there were no relations between *Acetobacter* and the lactic and acetic acid contents. *Aeriscardovia* belongs to Bifidobacteriaceae and may produce more acetic acid than lactic acid. The high acetic acid content found in bunkers A, C and E could be because of the activity of *Aeriscardovia*, indicating a potential new inoculant for whole crop corn silage. Unlike the findings in the guinea grass silage experiments, the integrated data for bacterial and fungal microbiota did not help improve the analysis of the fermentation patterns of whole crop corn silage.

This study has provided new insights into how bacterial and fungal microbiota interact in the ensiling process. The acetic acid fermentation found in tropical grass silage may be *Lactobacillus*-driven. Studies should be expanded for precise control and management of forage conservation.

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