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Bacterial and Fungal Microbiota of Guinea Grass Silage Shows Various Levels of Acetic Acid Fermentation

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Abstract: 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 °C) and high (40 °C) 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 the DC silage stored at 25 °C. The lactic acid reduction and acetic acid increase were suppressed in the DC silage stored at 40 °C. Increased abundances of *Lactobacillus*, *Clostridium*, and *Wallemia*, as well as decreased abundances of *Saitozyma*, *Papiliotrema*, and *Sporobolomyces* were observed in DC silages from day three 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 were the most prevalent bacteria in DC (38.1–64.9%) and WT (50.9–76.3%) silages, and their 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.

Keywords: bacteria; fungi; silage; storage temperature; tropical grass

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1. Introduction

Microbiota analysis using DNA information has become more sophisticated and easily accessible in the past 20 years [1]. Many studies have been conducted to elucidate the microbiota involved in fermentation and the 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 the 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 [2].

The acetic acid fermentation found in tropical grass silages is also a topic for which the mechanisms and control procedures have not been clarified. Due to the fact that acetic acid fermentation is inevitable even with molasses addition, the lack of soluble sugars is unlikely to be the main factor [3]. In addition, acetic acid is 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 [3–5]. 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 [6], but 1,2-propanediol and its metabolite, 1-propanol have not been found in large amounts in tropical grass silages. *Acetobacter* spp., which produce acetic acid through oxidizing ethanol, are rarely detected, and their metabolism under aerobic conditions does not explain the decrease in lactic acid levels [7].

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 anaerobic 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 [8]. 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.

2. Materials and Methods

2.1. 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 °C for 3 days, and for 1 and 2 months.

2.2. Chemical Component and Fermentation Characteristic Analyses

The dry matter (DM) contents of pre-ensiled and silage samples were determined at 60 °C 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 [9]. 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 °C. The ammonia nitrogen content was determined using the phenol–hypochlorite reaction.

2.3. MiSeq Preparation

Two silages were chosen at random from triplicate silages and subjected to DNA extraction and subsequent MiSeq analyses [10]. 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 [11], 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 [12]. 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 [13]. A two-round polymerase chain reaction (PCR) was performed to reduce any potential bias of the MiSeq adaptor overhang [14]. For the V4 region of bacterial 16S rRNA genes, the PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. For the ITS2 region of the fungal rRNA genes, the following conditions were employed: 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, and a final extension step at 72 °C 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 °C for 2 min, 10 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s, and one cycle at 72 °C 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.

2.4. Bioinformatics Analysis

Bioinformatics analysis was performed using the QIIME2 program [15]. 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 [16] and an unmerged ITS2 sequence from a fungal amplicon sequencing dataset was extracted for quality control. DADA2 [17] 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 [18], that is, 100% operational taxonomic units (OTUs), were aligned using the MAFFT program plugin via q2-alignment [19] to construct a phylogenetic tree with FastTree2 via q2-phylogeny [20]. 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 [21]. 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.

2.5. 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. The 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., Armonk, NY, USA).

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 [22].

3. Results

3.1. 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 °C for 1 and 2 months (Table 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 1. Dry matter, pH value, and fermentation products of direct-cut guinea grass silage stored at 25 and 40 °C 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 °C	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 °C	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 °C separately.

Intensive lactic acid fermentation was observed during the initial ensiling of DC silage stored at 40 °C; the ratio of lactic acid to acetic acid was >3.0 on day three. Unlike the DC silage stored at 25 °C, 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 °C, regardless of the ensiling period.

In WT silages stored at 25 and 40 °C, 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). 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.

Table 2. Dry matter, pH value, and fermentation products of wilted guinea grass silage stored at 25 and 40 °C 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 °C	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 °C	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 °C separately.

3.2. 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 (Table S1). 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.

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 3; Figure 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 °C. 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 °C, 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 °C.

In WT silage, unclassified Enterobacteriaceae appeared more than that in DC silage stored at 25 °C; the abundance on day 3 was >60%, followed by that of *Lactococcus* (12.4%) in WT silage stored at 25 °C (Table 4). The abundance of unclassified Enterobacteriaceae was >70%, and *Enterococcus* (11.8%) was the second-most abundant on day three in WT silage stored at 40 °C. 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 3. Relative abundances (%) of the eight most abundant bacterial genera in direct-cut 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	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 °C	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 °C separately.

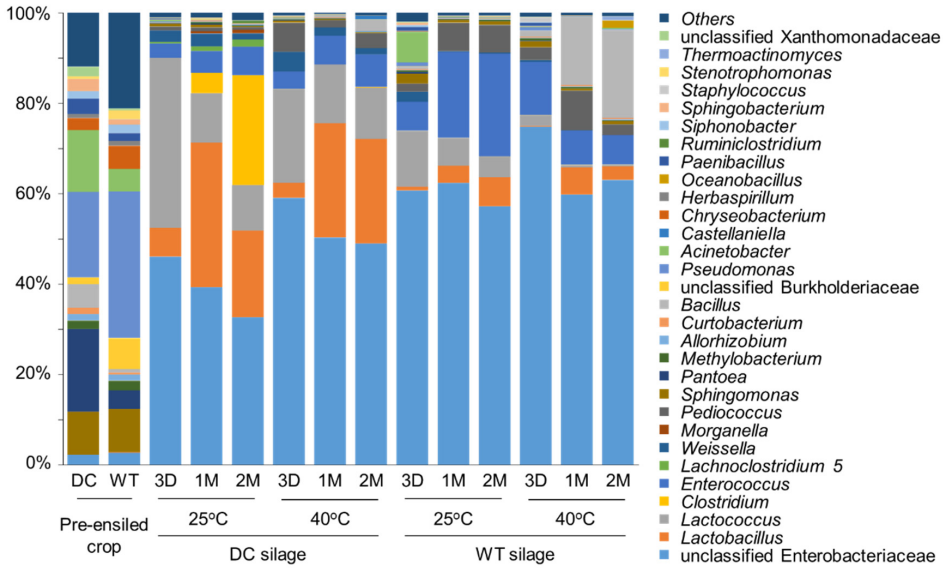

Figure 1. Relative abundances of the 30 major genera (>1% in abundance) of the bacterial microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. DC and WT indicate direct-cut and wilted crops and silages. 3D, 1M, and 2M indicate storage for 3 days, 1 month, and 2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

Table 4. 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). 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.

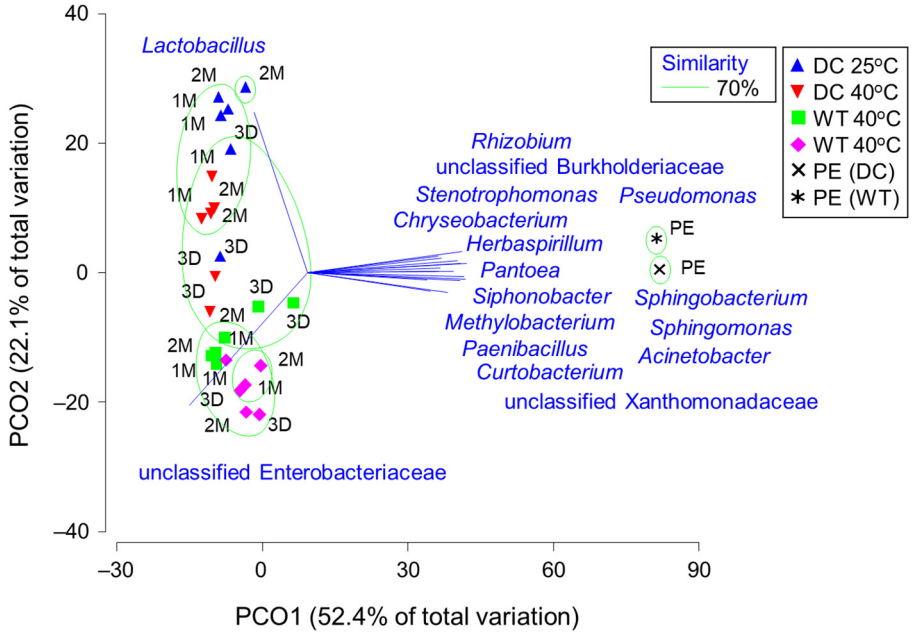

Figure 2. Beta diversity of the bacterial microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance > 1%). Points that are closer together 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.

3.3. 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 (Table S2). 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 °C, 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 °C.

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 °C, the abundance of *Wallemia* decreased on day three but increased again as ensiling was extended (Table 5; Figure 3). In DC silage stored at 40 °C, *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.

Table 5. 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.

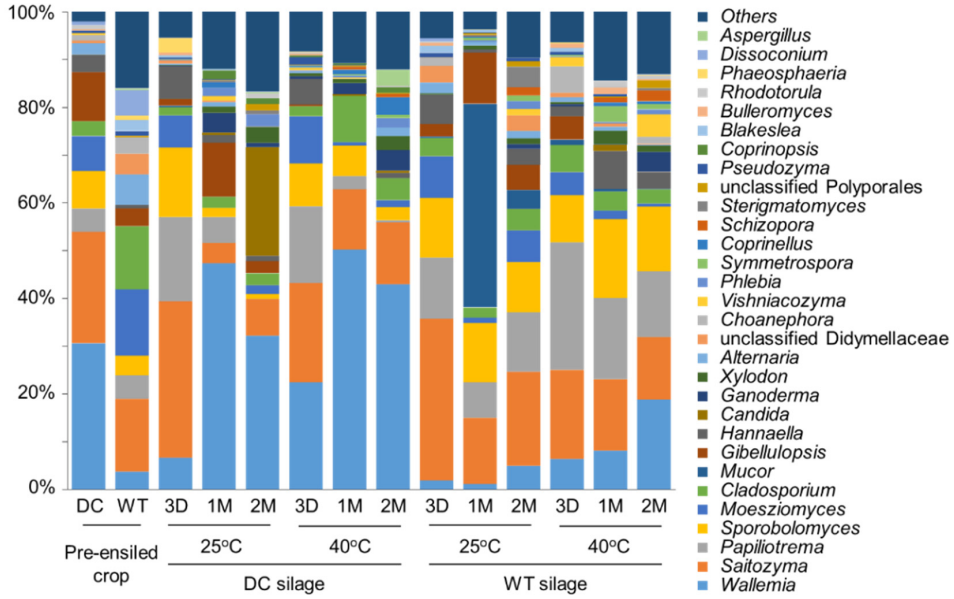

Figure 3. Relative abundances of the 30 major genera (>1% in abundance) of the fungal microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. DC and WT indicate direct-cut and wilted crops and silages. 3D, 1M, and 2M indicate storage for 3 days, 1 month, and 2 months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

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%) (Table 6). Similar to DC silage, a temporal increase in *Saitozyma* on day three was observed in the WT silage. 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*.

The PCoA plots did not clarify the differences in fungal microbiota between the pre-ensiled crop, DC silage, and WT silage (Figure 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 °C for 1 month, was separately characterized by *Mucor*.

Table 6. Relative abundances (%) of the eight most abundant fungal genera in wilted 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	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 °C	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 °C separately.

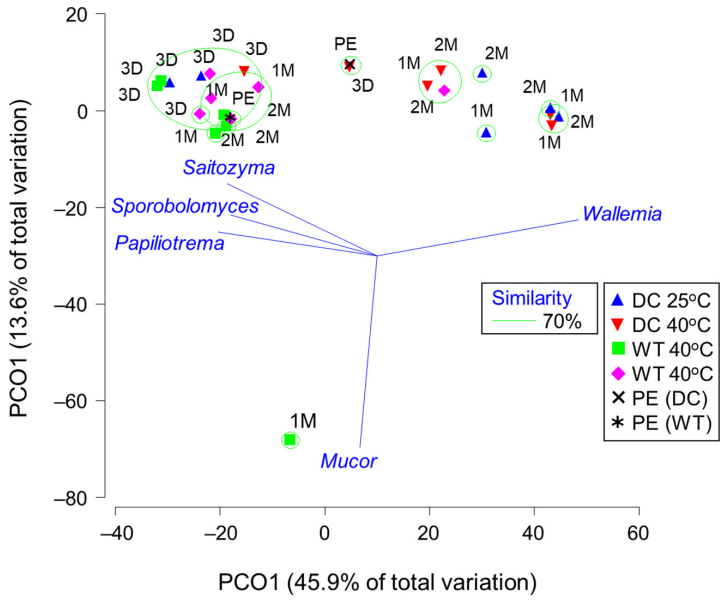

Figure 4. Beta diversity of the fungal microbiota of pre-ensiled crops and silages prepared from direct-cut and wilted guinea grass. The principal coordinate analysis plot of the Bray-Curtis distance matrix was generated from the filtered genus (relative abundance >1%). Points that are closer together 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.

3.4. 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 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.

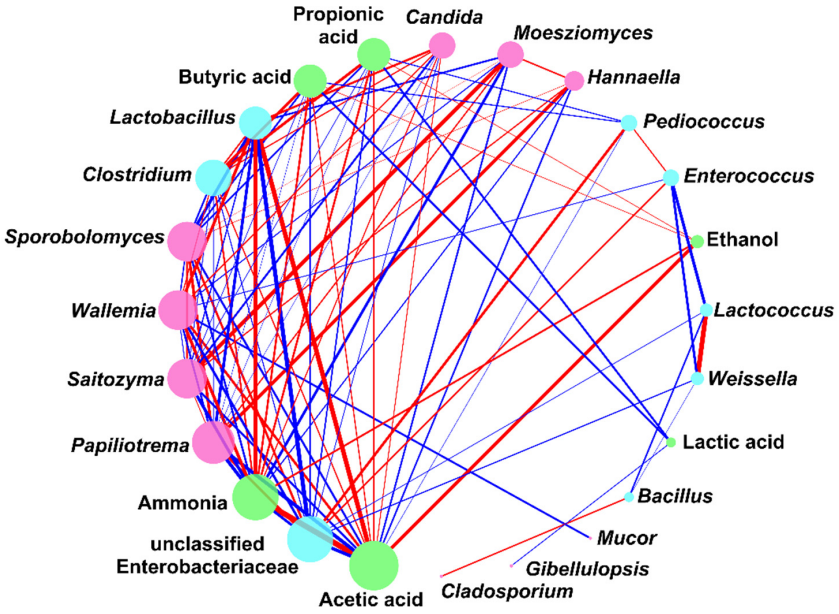

Figure 5. Correlation network diagram for fermentation products and major bacterial and fungal genera of direct-cut and wilted guinea grass silage. Green, blue, and purple nodes represent the fermentation product, bacterial genus, and fungal genus, respectively. The node size represents the degree of 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.

4. 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 °C and 40 °C storage. In DC silage stored at 25 °C, 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 [3,5]. *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 [5]. 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 [6], 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 [23]. *Veillonella*, *Selenomonas*, and *Megasphaera* are well-known lactate-utilizing and acetate-producing bacteria [23], 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. [24] 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 [24]. 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 [8], 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 [25]. 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 [26], 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 [27,28]. Likewise, *Sporobolomyces* are commonly found in low-nutrition environments [29]. 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 °C) were observed in DC silage. At 25 °C, butyric acid increased along with acetic acid production, whereas at 40 °C, butyric acid was almost completely suppressed, and acetic acid was reduced to about half of that at 25 °C. Usually, silage is more susceptible to clostridial fermentation during warm storage when compared to cold storage [30]. In addition, ensiling at a high temperature lowers lactic acid content due to a shift from homolactic to heterolactic fermentation [31]. Meanwhile, ensiling at a substantially high temperature, such as at about 40 °C, 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 [32]. Li et al. [24] found higher lactic acid production at 40 °C than at 28 °C 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. [33] found that, although acetic acid was predominant in Napier grass silage at 30 °C, lactic acid became prevalent when stored at 40 °C. In this study, storage at 40 °C 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 °C compared to 25 °C. 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 [34,35]. In WT silage stored at 40 °C, 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.

5. Conclusions

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.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8010010/s1.

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