NEW MICROBIAL INOCULANTS FOR ENHANCING FERMENTATION QUALITY OF SILAGE

EDITED BY: Fuyu Yang, Yimin Cai, X. S. Guo and Jin Zhong
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NEW MICROBIAL INOCULANTS FOR ENHANCING FERMENTATION QUALITY OF SILAGE

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Effects of Citric Acid and *Lactobacillus plantarum* on Silage Quality and Bacterial Diversity of King Grass Silage

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To better understand the mechanism underlying the citric acid (CA)-regulated silage fermentation, we investigated the bacterial community and fermentation quality of king grass (KG) ensiled without (CK) or with *Lactobacillus plantarum* (L), CA and the combination of L and CA (CAL). The bacterial community was characterized by using the 16Sr DNA sequencing technology. The L and CA treatments altered the silage bacterial community of KG, showing reduced bacterial diversity, while the abundance of desirable genus *Lactobacillus* was increased, and the abundances of undesirable genus *Dysgonomonas* and *Pseudomonas* were decreased. The additives also significantly raised the lactic acid content, dropped the pH, and reduced the contents of acetic acid, propionic acid, and ammonia-N in ensiled KG ($P < 0.01$). Besides, the combination treatment was more effective on silage fermentation with the highest pH and lactic acid content, while the contents of acetic acid, propionic acid, and ammonia-N were the lowest ($P < 0.01$). Moreover, CAL treatment exerted a notable influence on the bacterial community, with the lowest operational taxonomic unit (OTU) number and highest abundance of *Lactobacillus.* Furthermore, the bacterial community was significantly correlated with fermentation characteristics. These results proved that L and CA enhanced the KG silage quality, and the combination had a beneficial synergistic effect.

Keywords: king grass, *Lactobacillus plantarum*, citric acid, silage fermentation, bacterial community

INTRODUCTION

As a *Pennisetum* grass species, king grass (*Pennisetum purpureum* Schumacher × *P. glaucum* (Linnaeus) R. Brown, KG) is widely distributed in the tropical and subtropical regions worldwide (Zhao et al., 2019). KG is a multifunctional plant, and it is extensively used in ecological environmental protection, bioenergy industry, and animal husbandry (Li et al., 2014; Li M. et al., 2019). KG vigorously grows in the summer or rainy season. However, its growth lags in the winter or drought season, resulting in biomass shortages (Li et al., 2014). Due to its seasonal harvest, KG should be properly preserved to provide a continuous supply. Ensiling is an approach for the long-term preservation of green forage. However, it is hard to make high-quality silage since KG or other

Pennisetum grass has a higher lignin content and a lower content of soluble carbohydrates (Li et al., 2014; Desta et al., 2016).

The fermentation quality of silage mainly depends on the microbial community and its metabolites. Therefore, further study on the composition of silage microbial communities can provide a valuable scientific basis to enhance fermentation quality (Guo et al., 2018). Previous studies have investigated the silage microbial community composition of many forages, such as Alfalfa, corn, Napier grass, and so on (Guo et al., 2018; Xu et al., 2019; Yuan et al., 2019). Besides, the mechanism underlying the additive-improved fermentation quality has been analyzed, and various additives can enhance the silage fermentation quality in different ways (Guo et al., 2018; Xu et al., 2019; Yuan et al., 2019; Li F. et al., 2020).

Nevertheless, few reports have investigated the microbial composition of KG silage, and the mechanism underlying the response to additives remains unclear. Citric acid (CA) is a type of antioxidant, and it is also a safe additive used in the food industry (Li M. et al., 2020). Besides, CA can be used as a carbohydrate source to provide energy to lactic acid bacteria (LAB) and accelerate their growth (Li et al., 2016). Therefore, CA is considered as an ideal and novel silage additive to enhance the fermentation quality (Li et al., 2016; Ke et al., 2017, 2018; He et al., 2019b; Li M. et al., 2020; Lv et al., 2020). Our previous study has found that CA improves the silage quality though increasing the abundances of lactic acid-producing microorganisms, *Paenibacillus* and *Bacillus* (Li M. et al., 2020). Meanwhile, Lv et al. (2020) have reported that the addition of CA can increase the abundances of *Pediococcus* and *Lactobacillus*, while the abundances of *Enterobacter*, *Escherichia-Shigella*, and *Pantoea* are decreased, leading to enhanced *Amomum villosum* silage quality. However, the impacts of CA on the diversity of KG silage microorganisms remain largely unexplored.

In the present study, we hypothesized that LAB and CA had beneficial effects on the fermentation and microbes of KG silage, and their mixture might be shown a potential synergetic effect. Therefore, we aimed to assess the effects of LAB and CA on the microbial diversity and fermentation property of KG silage.

MATERIALS AND METHODS

Silage Preparation

King grass (Reyan No. 4) was grown in the experimental base of the Chinese Academy of Tropical Agricultural Sciences (109°58′E, 19°52′N). The first cut KG of the vegetative stage (approximately 1.5–1.8 m height) was harvested and chopped into about 2-cm pieces by grass shredding machine (Donghong No. 1, Donghong Mechanical Equipment Co., Ltd., China). Four different treatments were conducted in our current study as follows: control (no additive, CK), *Lactobacillus plantarum* (L), CA, and the combination of *L. plantarum* and CA (CAL). According to the manufacturer's guidelines, the additives were dissolved in sterile distilled water and then mixed thoroughly with the grass. An equal amount of sterile distilled water was added to the control group. Every treatment was carried out in triplicate. The application rate of *L. plantarum* (Snow Brand Seed Co., Ltd., Japan) and CA (Sinopharm Chemical Reagent Co., Ltd., China) was 1.0×10^5 colony-forming units (cfu)/g of fresh matter (FM) and 5 g/kg of FM, respectively. Briefly, 200 g of KG was blended with additives, and the mixture was placed in plastic bags (35 cm × 12 cm × 5 cm; Guozhong Packing Co., Ltd., Haikou, China). A total of 60 bags (four treatments × five ensiling durations × three replicates) were prepared and stored at normal temperature (25–30°C). Three bags were used to determine the chemical composition and organic acid on days 1, 3, 7, 14, and 30. The microbial community was determined after 30 days of fermentation.

Chemical Composition, Fermentation, and Microbial Analysis

Specimens were heated at 65°C for 72 h to determine the dry matter (DM) content, and dried materials were ground for chemical analysis. Crude protein (CP) and ether extract (EE) were determined according to the Guidelines of the Association of Official Analytical Chemists (AOAC 1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined as previously described by Van Soest et al. (1991). Water soluble carbohydrate (WSC) was determined according to a previously described method (Murphy, 1958). The fermentation quality of silage was determined using distilled water extracts. Briefly, 50 g wet silage was blended with 200 mL distilled water, followed by incubation at 4°C for 24 h and then filtration. The pH and concentrations of lactic acid, acetic acid, propionic acid, butyric acid, and ammonia-N were measured as previously established (Li et al., 2014). Microbial counts were analyzed using the plate count method on MRS agar, Violet Red Bile agar, and Rose Bengal agar as previously described (Li P. et al., 2019).

Microbial Community Analysis

The above-mentioned extracts were used for the molecular analysis of the microbiota. Briefly, 20 mL filtrate was centrifuged at 12,000 *g*/min for 5 min, and the sediment was collected from the bottom. Microbial DNA was isolated from silage specimens with the E.Z.N.A®. soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions. The concentration and purity of extracted DNA were assessed by NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States), and DNA integrity was confirmed by electrophoresis on 1% agarose gel. Primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were adopted to amplify the V3–V4 hypervariable regions of the bacterial 16S rRNA gene using a thermocycler PCR system (GeneAmp 9700, ABI, United States). After PCR products were purified and quantified, next-generation sequencing was carried out using Illumina MiSeq 2500 platform (Illumina, Inc., San Diego, CA, United States), and paired-end reads of 250 bp were generated.

The assembly of tags was carried out using filtered reads according to the principles as follows: overlap between paired-end reads should be more than 10 bp and less than 2% mismatch. The unique tags were obtained by removing redundant tags using software MOTHUR (Schloss et al., 2009). The abundance was

then determined using the resultant unique tags. The high-quality reads were grouped into operational taxonomic units (OTUs) defined at a similarity of 97%. Diversity metrics were determined using the core-diversity plug-in within QIIME2[1] (Callahan et al., 2016). The microbial diversity within an individual sample was assessed using the alpha diversity indices, including observed OTUs, Chao1 richness estimator, Shannon diversity index, and Faith's phylogenetic diversity index. Beta diversity was analyzed to assess the structural variation of microbiota across specimens, and then principal component analysis (PCA) was conducted (Vázquez-Baeza et al., 2013). Appropriate methods were employed to identify the bacterial strains with different abundances among samples and groups (Segata et al., 2011). Unless otherwise specified above, parameters used in the analysis were set as default. The heat map function of the R software[2] and genus information for the *Pennisetum sinese* silage were used to generate a heat map. The data were analyzed using the free online BMKCloud Platform.[3] The sequencing data were submitted to the National Center for Biotechnology Information Sequence Read Archive database under the BioProject accession number of PRJNA556187.

Statistical Analysis

The impacts of additives (*L. plantarum* and CA), ensiling duration, and their interactions were investigated by two-way analysis of variance using general linear model (GLM) procedure of SAS 9.3 software (SAS Institute Inc., Cary, NC, United States). Significant differences were compared using Duncan's multiple range tests, and $P < 0.05$ was considered statistically significant.

RESULTS

Chemical and Microbial Composition of Fresh KG

Table 1 shows the chemical and microbial compositions of fresh KG. The contents of DM, EE, CP, NDF, ADF, and WSC were 152.8, 60.2, 91.2, 765.3, 496.9, and 72.1 g/kg, respectively. Meanwhile, the number of LAB, yeast, and mold in fresh KG was 4.22, 2.78 and 3.04 cfu/g, respectively.

Chemical composition of KG during ensiling

Table 2 shows the chemical composition dynamics of KG silages during 30 days of ensiling, which was reduced when the ensiling duration was prolonged. The DM content of the CK group was the lowest compared with the other groups on day 30 ($P < 0.05$). The CP content in the CK and L groups was lower compared with the CA and CAL groups on days 14 and 30 ($P < 0.05$). The contents of NDF and ADF showed a similar reducing trend, although it was not significantly different from the CK group. Meanwhile, lower contents of NDF and ADF were found in the CA and CAL groups on day 30 ($P < 0.05$). Additionally, the ensiling duration (D) and additive treatment (T) remarkably affected the contents of DM, NDF, and ADF ($P < 0.01$), and the CP content was only influenced by D ($P < 0.01$). Besides, we also found a significant interaction between D and T for the contents of DM, NDF, and ADF ($P < 0.01$).

Fermentation Property of KG During Ensiling

Table 3 illustrates the fermentation characteristic dynamics of KG silage. The pH in all groups was dramatically reduced ($P < 0.05$) during the 7 days of fermentation, while the lowest pH was found on day 30. Meanwhile, the highest and lowest pH on day 30 were found in the CK group and CAL group ($P < 0.05$), respectively. The lactic acid content in all groups was remarkably elevated during the fermentation, while the highest lactic acid content was found in the CAL group on day 30 ($P < 0.05$). The acetic acid content of the CA and CAL groups was lower compared with the CK and L groups on day 30 ($P < 0.05$). In the present study, the content of acetic acid in the CK and L groups was greater compared with the CA and CAL groups, which led to the pH raised in the CK and L groups accordingly. The propionic acid content in all groups was not significantly changed during ensiling, and the lowest content was found in the CAL group on day 30 ($P < 0.05$). Butyric acid was not detected in all groups, indicating that KG was well preserved. The ammonia-N content in all groups was dramatically increased ($P < 0.05$) after 3 or 7 days of fermentation, while the highest and lowest ammonia-N contents were found in the CK group and CAL groups ($P < 0.05$), respectively.

Microbiota Community of KG Silage

A total of 958,785 raw reads and 910,947 raw tags were generated, after an average of 70, 971 clean tags and 68,123 effective tags was obtained in each silage sample.

Table 4 and **Figure 1** show the α-diversity of the bacterial community of silages. Additive treatment affected the Ace, Chao 1, Shannon, and Simpson indices of bacterial diversity (**Table 4**). For community richness comparison, the Shannon index was lower, and the Simpson index was higher in the additive-treated groups compared with the CK group. A total of 249 OTUs were detected, with the highest number of OTUs found in the CK group (234), and the lowest number found in the CAL group (195) (**Figure 1**). Venn analysis exhibited that the additive treatment resulted in 181 common OTUs, and there were 31, 3, and 2 special OTUs in the CK, CA, and L groups, respectively. The PCA was employed to examine the correlations among the community structures of the silage bacterial community. A clear separation and difference of bacterial communities were found in four groups (**Figure 2**), suggesting that the bacterial composition was changed with different additive treatments. Therefore, we drew a conclusion based on the α-diversity and β-diversity that the LAB and CA treatments could affect the bacterial diversity and community structure of KG silage.

[1]https://docs.qiime2.org/2019.1/

[2]http://www.r-project.org/

[3]www.biocloud.net

TABLE 1 | Chemical and microbial composition of KG.

	DM	g/kg (DM)						Log$_{10}$ cfu/g (FM)		
		OM	EE	WSC	CP	NDF	ADF	L	Yeast	Molds
King grass	152.8	898.4	60.2	72.1	91.2	765.3	496.9	4.22	2.78	3.04

DM, dry matter; OM, organic matter; EE, ether extract; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; L, lactic acid bacteria.

TABLE 2 | Chemical composition of KG silage.

Item	Treatment	Ensiling days					SEM	*P*-value		
		1	3	7	14	30		D	T	D*T
Dry matter (g/kg FW)	CK	158.6a	145.1b	134.6c	129.7cdB	124.8dB	2.31	<0.01	<0.01	<0.01
	L	160.2a	155.0b	147.3c	140.8dA	138.7dA				
	CA	156.8a	144.5b	140.8b	137.6bcA	135.1cA				
	CAL	159.3a	156.9a	146.1b	142.7bcA	138.8cA				
Crude protein (g/kg DM)	CK	91.4a	88.3a	86.8ab	83.4bB	82.5bB	0.79	<0.01	0.62	0.05
	L	93.7a	90.5a	87.4ab	84.6abB	82.3bB				
	CA	93.1a	92.1a	91.5a	90.8abA	88.9abA				
	CAL	92.5a	91.9a	91.3a	90.5abA	88.6abA				
Neutral detergent fiber (g/kg DM)	CK	767.7	762.0	759.9A	754.1A	750.7A	10.32	<0.01	<0.01	<0.01
	L	771.3a	750.3ab	730.8bAB	720.3bB	701.5bB				
	CA	767.5a	749.85ab	714.9bB	661.4bC	633.8bC				
	CAL	778.2a	738.12ab	705.4bB	656.8bC	634.3bC				
Acid detergent Fiber (g/kg DM)	CK	490.4	488.2	481.7A	477.2A	474.4A	13.85	< 0.01	< 0.01	< 0.01
	L	497.6a	476.3a	450.0abB	436.4abB	421.6bB				
	CA	500.5a	488.6ab	442.9bB	393.5bB	357.7cB				
	CAL	489.4a	461.09ab	392.3bC	332.3cC	275.9dC				

*CK, control; L, Lactobacillus plantarum; CA, Citric acid; CAL, Citric acid + Lactobacillus plantarum; FM, fresh matter; DM, dry matter; ND, not detected; "–", default.D, ensiling days; T, treatment; D*T, interaction of ensiling days and treatment; SEM, standard error of means;Means with different letters in the same row (a–d) or column (A–D) differ (P < 0.05).*

Figure 3A describes the bacterial community at the phylum level. *Firmicutes* and *Proteobacteria* were dominant in all groups, *Bacteroidetes* was sub-dominant in the CK and CA groups, and *Cyanobacteria* was sub-dominant in the L group. The silage bacterial community was shifted upon the additives, the abundance of *Firmicutes* in additive treatment groups was increased, while the abundances of *Proteobacteria* and *Bacteroidetes* were decreased (except for the CA group) compared with the CK group. To further investigate the effects of additives on the bacterial community during ensiling, we examined the bacterial structures of KG silage at the genus level (**Figure 3** B). *Lactobacillus* was predominant in the four groups. The sub-dominant microbes, in turn, were *Dysgonomonas*, *Morganella*, and *Pseudomonas* in the CK group, *Gluconacetobacter* in the L group, *Pseudomonas* and *Gluconacetobacter* in the CA group, and *Pseudomonas* in the CAL group. The abundance of *Lactobacillus* was increased along with the additive treatments, while the abundances of *Dysgonomonas* and *Pseudomonas* were decreased (except for the CA group). Particularly, CA treatment promoted the abundance of *Lactobacillus*, especially for the combination group, which accounted for as high as 95% of the total population.

The linear discriminant analysis (LDA) effect size (LEfSe) method was used to assess the differences in microbial community between four groups and explore the specific bacterial in each group (LDA score >4.0). **Figure 4** shows that CA exerted a dramatic impact on the microbial community. *Lactobacillus paraplantarum* and *Lactobacillus brevis* were the most abundant species in the CK group, and *Lachnospiraceae* and *Ruminococcaceae* were the most abundant families in the CA group, which could be the biomarkers of different treatments.

To better understand the silage fermentation process, we also assessed the correlation between the fermentation characteristics and the bacterial community of KG silage (**Figure 5**). The lactic acid was positively correlated with genera *lactobacilli*, *Weissella*, and *Enterobacter* ($P < 0.05$), while it was negatively correlated with genera *Delftia*, *Gemmobacter*, *Azospirillum*, and *Comamonas* ($P < 0.05$). The acetic acid was positively associated with genera *Ralstonia* ($P < 0.001$), while it was negatively associated with genera *Delftia*, *Gemmobacter*, *Brevundimonas*, *Sphingobacterium*, and *Azospirillum* ($P < 0.01$). The propanoic acid was highly and positively correlated with genera *Ralstonia* ($P < 0.001$), but negatively associated with genera *Delftia, Brevundimonas,* and *Comamonas* ($P < 0.05$). The pH was negatively correlated with genera *Lactobacillus*, *Weissella*, *Delftia*, *Azospirillum,* and *Clostridium* ($P < 0.05$). Moreover, the ammonia-N content was positively correlated with genera

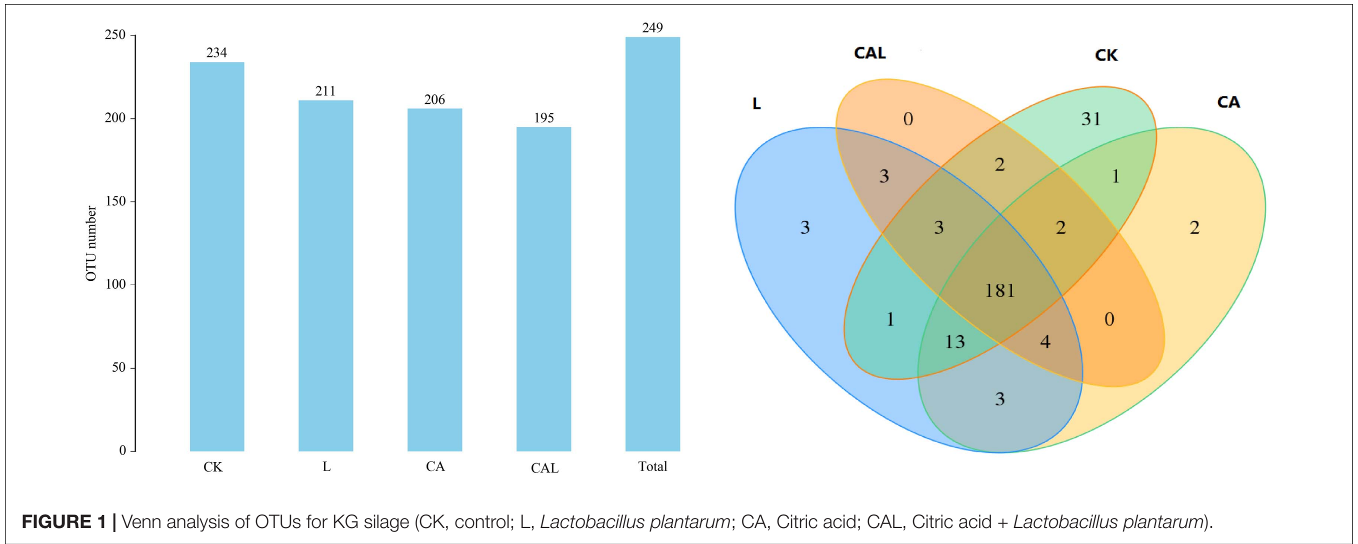

FIGURE 1 | Venn analysis of OTUs for KG silage (CK, control; L, *Lactobacillus plantarum*; CA, Citric acid; CAL, Citric acid + *Lactobacillus plantarum*).

FIGURE 2 | Beta-diversity of the bacterial community of KG silage (CK, control; L, *Lactobacillus plantarum*; CA, Citric acid; CAL, Citric acid + *Lactobacillus plantarum*).

TABLE 3 | Fermentation quality of ensiled KG.

Item	Treatment	Ensiling days					SEM	*P*-value		
		1	3	7	14	30		D	T	D*T
pH	CK	5.31a	4.91ab	4.78b	4.64bc	4.51cA	0.07	<0.01	<0.01	<0.01
	L	5.24a	4.84ab	4.54b	4.36bc	4.29cB				
	CA	4.91a	4.68ab	4.53b	4.44b	4.23cB				
	CAL	4.90a	4.75ab	4.54b	4.25bc	3.95cC				
Lactic acid (g/kg DW)	CK	22.8c	24.7cB	31.1bB	33.5abB	36.5aB	2.28	<0.01	<0.01	<0.01
	L	24.2c	26.0bcB	29.0bB	32.8abB	36.9aB				
	CA	21.1c	24.7cB	28.4bcB	34.4bB	41.1aB				
	CAL	27.4d	30.7cdA	37.9cA	50.9bA	63.7aA				
Acetic acid (g/kg DW)	CK	5.7c	11.3bc	16.3bA	24.4abA	27.6aA	1.65	<0.01	<0.01	<0.01
	L	5.7c	9.5bc	13.6bAB	20.5abAB	26.5aA				
	CA	5.9c	8.9bc	11.9bAB	18.1abAB	22.4aB				
	CAL	6.0c	9.6bc	12.1bAB	18.8abAB	23.5aB				
Propionic acid (g/kg DW)	CK	0.93A	0.88A	1.02A	0.85A	0.84A	0.05	<0.01	<0.01	<0.01
	L	1.15A	1.07A	1.02A	1.13A	0.92A				
	CA	0.82A	0.89A	1.01A	0.96A	0.70A				
	CAL	0.58B	0.62B	0.54B	0.48B	0.45B				
Butyric acid (g/kg DW)	CK	ND	ND	ND	ND	ND	–	–	–	–
	L	ND	ND	ND	ND	ND				
	CA	ND	ND	ND	ND	ND				
	CAL	ND	ND	ND	ND	ND				
Ammonia-N (g/kg DW)	CK	24.2c	28.5bcA	33.3bA	41.8abA	44.6aA	1.66	<0.01	<0.01	<0.01
	L	20.2b	24.6bAB	30.2abAB	32.6abB	36.5aB				
	CA	18.6b	22.4abAB	25.4abAB	28.7aB	30.9aC				
	CAL	18.1b	20.8abAB	21.5abB	22.6aBC	23.2aD				

*CK, control; L, Lactobacillus plantarum; CA, Citric acid; CAL, Citric acid + Lactobacillus plantarum; DM, dry matter; ND, not detected; "–", default. D, ensiling days; T, treatment; D*T, interaction of ensiling day and treatment; SEM, standard error of means; Means with different letters in the same row (a-d) or column (A-D) differ (P < 0.05).*

TABLE 4 | Alpha-diversity of bacterial diversity of KG silage.

	OTU	Chao1	ACE	Shannon	Simpson	Coverage
CK	234	202.52	197.86	2.57	0.17	0.99
L	211	196.71	197.34	2.46	0.19	0.99
CA	206	186.24	178.33	2.16	0.19	0.99
CAL	195	195.78	194.08	2.06	0.26	0.99

CK, control; L, Lactobacillus plantarum; CA, Citric acid; CAL, Citric acid + Lactobacillus plantarum.

Lactobacillus and *Ralstonia* ($P < 0.05$), while it was negatively associated with genus *Delftia* ($P < 0.05$).

DISCUSSION

Compared with the previous reports, the DM of KG was lower, while the contents of EE, CP, NDF, and ADF were higher (Li et al., 2014; Li M. et al., 2019; Xu et al., 2019). Recently, Li D. et al. (2019); Li P. et al. (2019) have found that the contents of DM and CP are 239 and 133 g/kg in KG, respectively, which are much higher compared with the above-mentioned studies. The differences in forage quality may be caused by cultivation, climatic conditions, soil fertility, growth period, and harvest time. The WSC content is a key factor affecting the fermentation quality. The WSC content in KG was 72.1 g/kg, which met the minimum requirement (60–80 g/kg DM) for well-conserved silage (Smith, 1962). However, the tropical forages normally have coarse and stemmy structures and high fiber fraction, which can cause the bad quality of silage (Yu et al., 2011; Li et al., 2017). Some previous studies have confirmed that KG ensiled alone can not achieve high fermentation quality (Li et al., 2014; Li M. et al., 2019). The well-preserved silage needs an LAB number of more than 5.0 $\log_{10}$ cfu/g during ensiling (Cai et al., 1998). The lower LAB number and higher yeast and mold contents indicated that more LAB was required for preparing KG silage. Moreover, the undesirable yeast and mold could be inhibited by the low pH, leading to the wide application of organic acid in forage silage (Guo et al., 2018; He et al., 2019b). Therefore, it might be helpful to achieve higher silage quality by rapidly reducing pH and promoting fermentation through adding CA and *L. plantarum*.

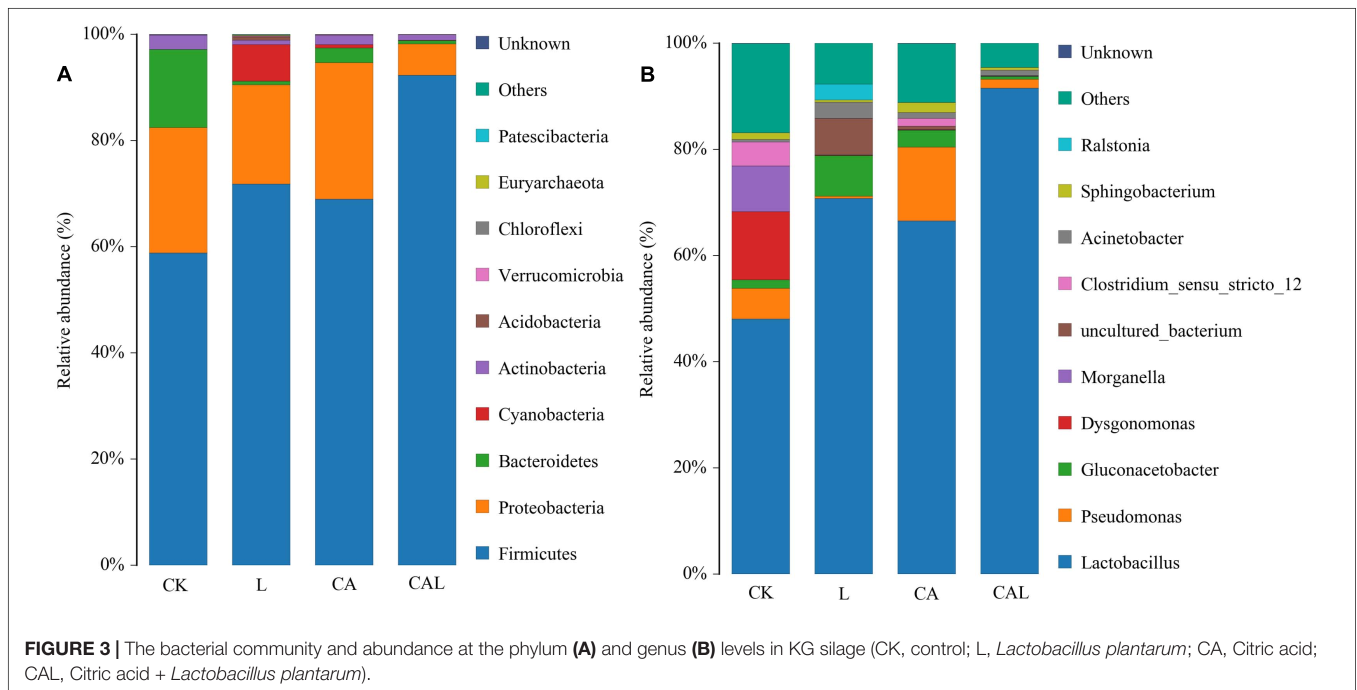

FIGURE 3 | The bacterial community and abundance at the phylum **(A)** and genus **(B)** levels in KG silage (CK, control; L, *Lactobacillus plantarum*; CA, Citric acid; CAL, Citric acid + *Lactobacillus plantarum*).

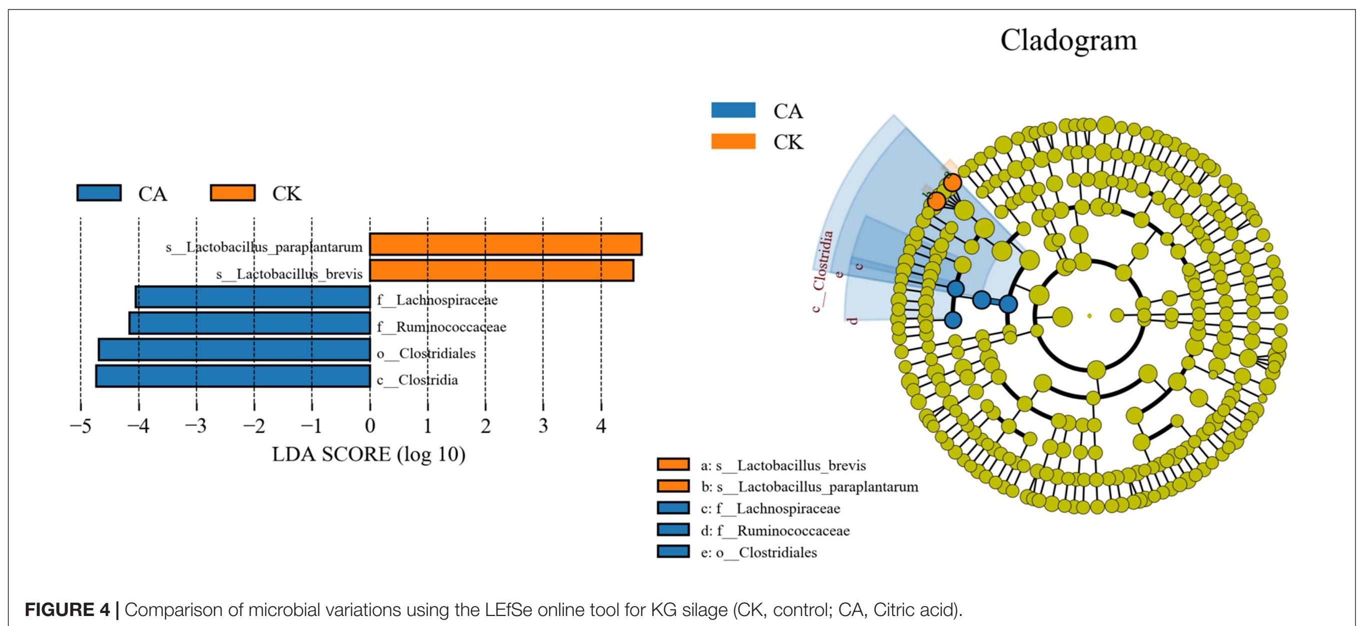

FIGURE 4 | Comparison of microbial variations using the LEfSe online tool for KG silage (CK, control; CA, Citric acid).

In the present study, the CA and CAL groups contained higher CP content. This finding might be explained as the effect of CA, which could inhibit protein hydrolysis (Ke et al., 2018). The CA treatment dramatically reduced the contents of NDF and ADF in KG. However, the effect of CA on ADF and NDF was contrary to the report of Ke et al. (2018) in alfalfa silage. The divergence might be related to the different types of forage that KG in this study was Gramineae plant, while the alfalfa was Leguminosae plant, which contained a higher amount of structural fibers. This probably impacted the effect of CA on ADF and NDF during ensiling. Meanwhile, the positive effect of CA on forage chemical composition has been also reported in previous studies (Li et al., 2016; Ke et al., 2017, 2018; He et al., 2019b). These results suggested that a combination of *L. plantarum* and CA could improve the feeding value of KG to some extent.

Silage pH plays an important role in the evaluation of fermentation property, and silage with pH 4.2 or lower would be considered as well-fermented silage (Edwards and McDonald, 1978). In this study, the pH value of the additive-treated groups was close to or below 4.2, which could ensure good preservation of KG silage. Li et al. (2016) and He et al. (2019b) have shown a similar pH of CA-treated silage. In contrast, Ke et al. (2017, 2018)

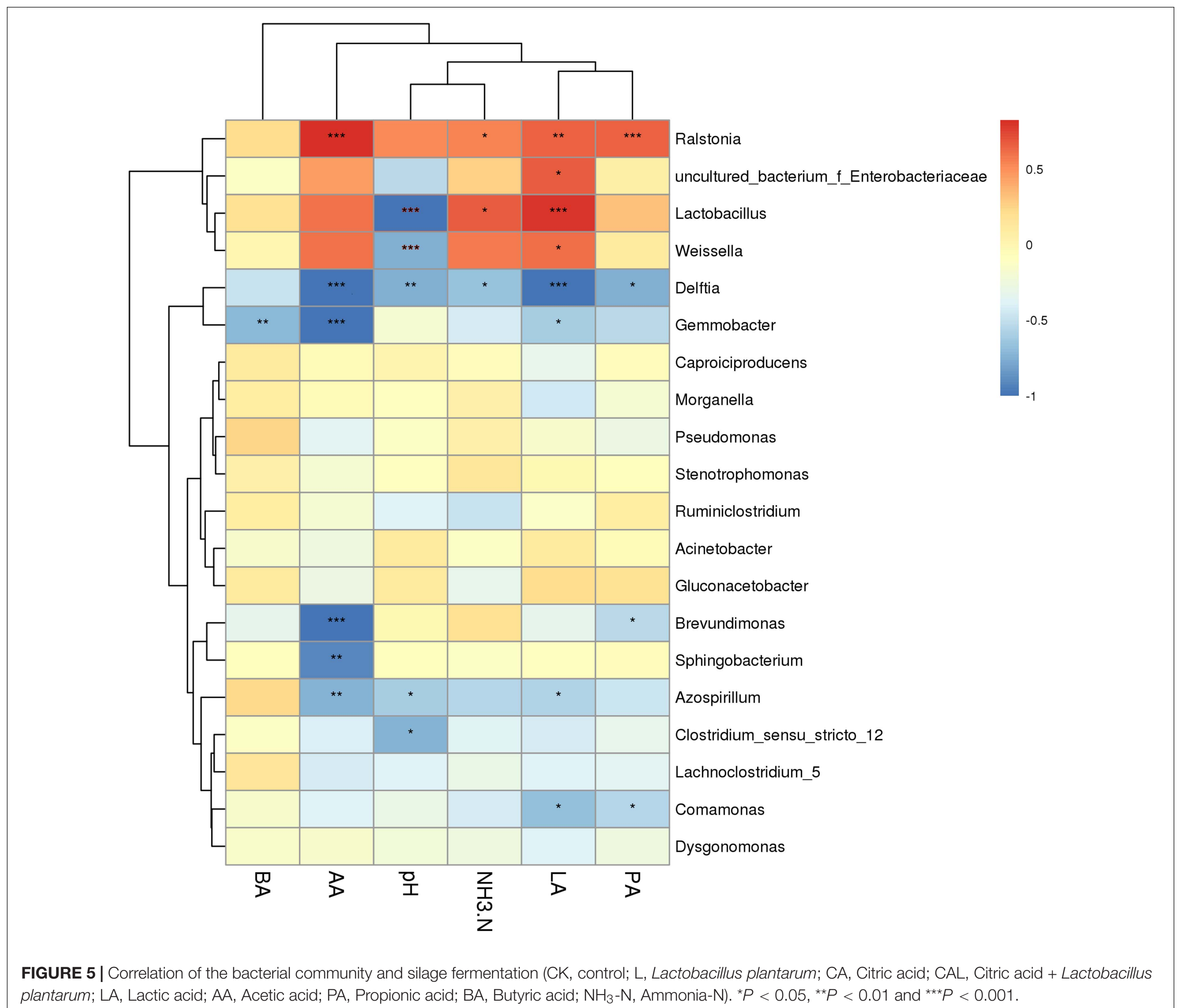

FIGURE 5 | Correlation of the bacterial community and silage fermentation (CK, control; L, *Lactobacillus plantarum*; CA, Citric acid; CAL, Citric acid + *Lactobacillus plantarum*; LA, Lactic acid; AA, Acetic acid; PA, Propionic acid; BA, Butyric acid; NH_3-N, Ammonia-N). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

have shown a higher pH value in CA-treated alfalfa silage, which is even higher than 5.0, and such discrepancy may be attributed to the different application rates. The lactic acid in silage is the dominant fermentation product, which is another important evaluation index of silage quality. The addition of CA directly decreased the pH, which could inhibit harmful microorganisms and promote the reproduction and growth of LAB, leading to increased lactic acid content. Such finding was consistent with the previous data, indicating that CA can increase the lactic acid content, while different supplementation levels have various effects (Ke et al., 2017; He et al., 2019b). However, some studies have demonstrated the different effect. Li et al. (2016) and He et al. (2019b) have reported that CA treatment reduces lactic acid content in alfalfa and *Neolamarckia cadamba* leaf silage. Zhao et al. (2004) and Seo et al. (2007) have shown that released CA can be used by some yeast strains as a carbon and energy sources, and the growth of yeasts may be more competitive than LAB when an appropriate level of CA is applied, resulting in the lower lactic acid content. Therefore, the optimal amount of CA in different silage materials needs to be further studied. As the main metabolite of *Acetobacter* fermentation in silage, acetic acid is also a crucial index for the evaluation of silage quality. The higher acetic acid concentration always leads to a higher pH, which benefits the undesirable microorganism *Clostridia*, leading to reduced silage quality (Zheng et al., 2017). The ammonia-N content in silage often reflects the breakdown efficiency of protein to peptide. The comparatively low ammonia-N content in additive-treated silage could be attributed to the effect of lower pH values, indicating that the activity of protease was inhibited and preservation of more nutrients. The above-mentioned results indicated that the addition of *L. plantarum* and CA in the ensiling process could promote the fermentation quality, and the combination treatment more efficiently enhanced the fermentation. He et al. (2019b) have

reported the profitable associated effects of *L. plantarum* and CA combination treatment on alfalfa silage. Similarly, Guo et al. (2018) have also found that the combination of hexanoic acid and *L.plantarum* can improve the fermentation characteristics compared with the single treatment in Napier grass silage.

In our study, the CK group showed the high Shannon, Ace, and Chao1 indices, as well as the low Simpson index mean high bacterial diversity. These results implied that additive treatment could reduce bacterial diversity. It could be explained that the LAB and CA treatment decreased the pH, inhibited the growth of harmful microorganisms, and promoted the growth of LAB species. When LAB became the dominant species, the bacterial diversity was decreased. Similar data have been reported by Ni et al. (2017) and (Wang et al., 2019a,b) in soybean, *Moringa oleifera* leaves and *Morus alba* leaves. Meanwhile, PCA indicated that a clear separation and difference of bacterial communities were found in different groups of KG silage, suggesting that the bacterial composition was changed in the ensiling process with different additive treatments. This could be explained by that CAs could provide additional substrates to LAB and accelerate its growth, while the addition of *L. plantarum* also increased desirable microorganisms.

In the present study, *Firmicutes* and *Proteobacteria* were the top two dominant phyla in KG silage, which was similar to previous studies (Xu et al., 2017; Dong et al., 2019). However, the total abundances of the two phyla were increased from approximately 80% to more than 95% after fermentation (Liu et al., 2019; Wang et al., 2019b). Such elevation might be attributed to different silage materials and treatments. Normally, as the major bacterial strain with desirable functions, the *Lactobacillus* is dominant in well-preserved forage silage, because it is responsible for driving lactic fermentation during ensiling (Bao et al., 2016; Ni et al., 2017, 2018; Guan et al., 2018; Liu et al., 2019). CA treatment also promoted the abundance of *Lactobacillus*, especially for the combination group, which accounted for as high as 95% of the total population. It could be attributed to that the addition of CAs could provide substrates and supplement energy to *Lactobacillus*, which was beneficial for its propagation. Besides, the acidic environment formed by the addition of CA also inhibited the undesirable microbes, which might be conducive to the growth of *Lactobacillus*. Similar data have also been found by Lv et al. (2020) that the addition of CA can increase *Pediococcus* and *Lactobacillus*, resulting in enhanced silage quality *of Amomum villosum*. However, our previous study has found that CA improves the silage quality through raising the abundances of *Paenibacillus* and *Bacillus,* and other studies have also shown that *Lactobacillus* is not the dominant bacterial strain in organic acid-treated silage (He et al., 2019a, 2020; Li M. et al., 2020). Therefore, the type of organic acids and the proportion of additives may affect the bacterial community. In contrast, *Dysgonomonas* is a facultative anaerobe, *Pseudomonas* is an aerobic bacterium, and both of them are undesirable microorganisms and can reduce the fermentation quality (Hofstad et al., 2000; Dunière et al., 2013). *Dysgonomonas* is rarely reported in silage, and its effect and mechanism in silage fermentation need to be further studied. *Pseudomonas* spp. is considered an undesirable bacterial strain for silage, because it may be associated with the production of biogenic amines, leading to decreased protein content and nutritional value (Dunière et al., 2013). In the present work, the LAB additive treatment markedly reduced the abundance of *Pseudomonas*, which was harmful to the silage quality and nutrient preservation. Similar findings have been reported in corn stover, alfalfa, *M. oleifera* leaves, and red clover (Xu et al., 2017; Ogunade et al., 2018; Dong et al., 2019; Wang et al., 2019b). *Gluconacetobacter* is widely used in the production of vinegar and wine, which is a type of acetic acid-producing bacterial strain, and can consume ethanol and sugar substances (Du et al., 2018; Huang et al., 2018). The higher abundance of *Gluconacetobacter* resulted in more acetic acid, leading to raised pH and impaired fermentation quality.

Silage fermentation is a very complex biological process involving a variety of microorganisms, and such a process produces many different metabolites during ensiling. *Lactobacillus* is the core microorganism during the ensiling process, *Weissella* is also widely distributed in silage, and both of them produce lactic acid and determine the silage quality (Cai et al., 1998). Similar to our findings, previous studies have reported that the concentration of lactic acid is positively correlated with genera *Lactobacillus* and *Weissella* in silage (Xu et al., 2017; Guan et al., 2018; Ogunade et al., 2018; Yang et al., 2019). Meanwhile, *Enterobacter* converts lactic acid to acetic acid and other organic acids, so it is positively correlated with lactic acid (Ostling and Lindgren, 2010). The ammonia-N is created by the synthetic effect of plant enzymes and microbial activity, and the reduction of silage ammonia-N was attributed to the rapid acidification of LAB. Therefore, *Lactobacillus* was sensitive to lower pH, contributing to the high correlation coefficients between ammonia-N and *Lactobacillus*. Our results were consistent with the other studies (Ogunade et al., 2018; Ren et al., 2019).

CONCLUSION

The addition of LAB or CA significantly raised lactic acid content, decreased pH, and reduced contents of acetic acid, propionic acid, and ammonia-N in ensiled KG. The LAB and CA treatment also altered the bacterial community of KG silage, which reduced bacterial diversity. However, such treatments increased the abundance of desirable *Lactobacillus*, but decreased the abundance of undesirable *Dysgonomonas* and *Pseudomonas*. Besides, their combination treatment displayed a beneficial synergistic impact on silage fermentation, and notable influence on the bacterial community. Furthermore, the bacterial community was significantly correlated with fermentation characteristics. Collectively, our results confirmed that LAB, CA, and their combination exerted beneficial effects on KG silage fermentation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://catalog.data.gov/dataset/sequence-read-archive-sra, PRJNA556187.

AUTHOR CONTRIBUTIONS

ML, XZ, YC, and RL did the experimental design work. ML, XZ, YC, RL, and JT conducted the experiments. ML, XZ, YC, RL, JT, and HZ analyzed the data. ML, XZ, and YC wrote the manuscript. All authors read and approved the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Succession of Bacterial Community During the Initial Aerobic, Intense Fermentation, and Stable Phases of Whole-Plant Corn Silages Treated With Lactic Acid Bacteria Suspensions Prepared From Other Silages

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The present study was aimed at investigating the bacterial community in lactic acid bacteria (LAB) suspensions prepared from whole-plant corn silage (LAB suspension-CS) and *Elymus sibiricus* silage (LAB suspension-ES) and the bacterial community succession of whole-plant corn silages inoculated with LAB suspension-CS or LAB suspension-ES during initial aerobic phase, intense fermentation phase, and stable phase. The LAB suspensions were cultured in sterile Man, Rogosa, Sharpe broth at 37°C for 24 h and used as inoculants for ensiling. The chopped whole-plant corn was treated with distilled water (CK), LAB suspension-CS (CSL), or LAB suspension-ES (ESL) and then ensiled in vacuum-sealed plastic bags containing 500 g of fresh forage. Silages were sampled at 0 h, anaerobic state (A), 3 h, 5 h, 10 h, 24 h, 2 days, 3 days, 10 days, 30 days, and 60 days of ensiling with four replicates for each treatment. The results showed that *Lactobacillus*, *Weissella*, and *Lachnoclostridium_5* dominated the bacterial community in LAB suspension-CS; *Lactobacillus* was the most predominant bacterial genus in LAB suspension-ES. During the initial aerobic phase (from 0 h to A) of whole-plant corn silage, the pH and the abundances of *Pantoea*, *Klebsiella*, *Rahnella*, *Erwinia*, and *Serratia* increased. During the intense fermentation phase (from A to 3 days), the pH decreased rapidly, and the microbial counts increased exponentially; the most predominant bacterial genus shifted from *Pantoea* to *Weissella*, and then to *Lactobacillus*; inoculating LAB suspensions promoted the bacterial succession and the fermentation process, and LAB suspension-CS was more effective than LAB suspension-ES. During the stable phase (from 3 to 60 days), the pH and the microbial counts decreased, and *Lactobacillus* dominated the bacterial community with a little

decrease. The results also confirmed the existence of LAB fermentation relay during fermentation process, which was reflected by *Weissella*, *Lactococcus*, and *Leuconostoc* in the first 5 h; *Weissella*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* between 5 and 24 h; and *Lactobacillus* from 24 h to 60 days.

Keywords: whole-plant corn silage, bacterial community succession, lactic acid bacteria suspension, initial aerobic phase, intense fermentation phase, stable phase, lactic acid bacteria fermentation relay

INTRODUCTION

Ensiling is an effective method for preserving moist forage crops and supplying quality forage to livestock throughout the year (Guan et al., 2018; Zhang et al., 2019). During the fermentation process, water-soluble carbohydrates are converted into organic acids by lactic acid bacteria (LAB) under anaerobic conditions to reduce pH and inhibit undesirable microorganisms for long-term preservation of silage (Keshri et al., 2018). Generally, the main LAB genera that play a major role in silages include *Lactobacillus*, *Weissella*, *Pediococcus*, *Lactococcus*, *Enterococcus*, and *Leuconostoc* (McGarvey et al., 2013; Muck, 2013; Gharechahi et al., 2017). Additionally, the ensiling fermentation process is highly complex involving many types of microorganisms (Xu et al., 2019), and the process can be divided into four main phases including the initial aerobic phase, intense fermentation phase, stable phase, and aerobic feed-out phase (Weinberg and Muck, 1996; Dunière et al., 2013; Ávila and Carvalho, 2019). Therefore, understanding the succession of microorganisms and the correlation between microorganisms and fermentation quality in different phases of fermentation may reveal the fermentation process and provide a scientific basis for modulation of silage fermentation.

Whole-plant corn silage is the most common forage for ruminant worldwide because of the good fermentation quality and high nutritional value (Khan et al., 2015; Zhang et al., 2019). In the past decade, the microbial communities during ensiling have become a research focus of silages with the development of next-generation sequencing technologies (Romero et al., 2018). Gharechahi et al. (2017), Keshri et al. (2018), and Xu et al. (2020) reported the dynamics of microbial community during the ensiling of whole-plant corn silages treated with LAB or collected from different locations. Guan et al. (2018) determined the bacterial community in corn silages prepared with farm bunker-silo in Southwest China. Other previous studies revealed the bacterial community in whole-plant corn silages inoculated with LAB (Xu et al., 2019; Zhang et al., 2019), and the bacterial and fungal communities in whole-plant corn silages after 5 days of aerobic exposure (Keshri et al., 2018). However, little is known regarding the successions of microbial community during the initial aerobic phase and the fermentation relay of LAB during entire fermentation of whole-plant corn silages.

The LAB was usually used as silage additives to promote the ensiling process and improve the fermentation quality of end-silages (Weinberg and Muck, 1996). Some recent studies indicated that adding inoculants (containing selected LAB strains) changed the microbial community by making LAB dominated microorganism as soon as possible (Keshri et al., 2018; Guan et al., 2020; Xu et al., 2020). Several authors have reported that LAB prepared from alfalfa is more effective in improving the fermentation quality of alfalfa silage than that from other forage sources (Ohshima et al., 1997; Wang et al., 2009; Denek et al., 2011). Moreover, Ali et al. (2020) found that inoculating epiphytic microbiota from red clover has a greater effect on improving microbial succession and fermentation quality of red clover silages than that from maize and sorghum. To the best of our knowledge, no studies have reported the microbial succession of whole-plant corn silages ensiled with LAB prepared from whole-plant corn silages and other silages. *Elymus sibiricus* is a tall-growing and perennial bunchgrass widely distributed in Europe, Asia, and North America (Klebesadel, 1969), and used as forage because of high yield potential and good quality (Wang et al., 2017). Moreover, *E. sibiricus* has become a grass for artificial grassland and been ensiled for ruminants in China (Sun et al., 2009; Li et al., 2016).

We hypothesized that inoculation of the LAB suspensions prepared from whole crop corn and *E. sibiricus silages* at ensiling may alter the bacterial community and their successions in whole crop corn silage. Thus, the objectives of this study were to determine the bacterial community in LAB suspensions prepared from whole-plant corn silage and *E. sibiricus* silage and to characterize the changes in the bacterial community during the initial aerobic phase, intense fermentation phase, and stable phase of the anaerobic fermentation process in whole-plant corn silage inoculated with two different LAB suspensions prepared from whole crop corn and *E. sibiricus* silages.

MATERIALS AND METHODS

Preparing LAB Suspension

The silage samples (about 500 g) for preparing LAB suspensions were collected from wrapped whole-plant corn silage (four randomly selected bales; density >700 kg/m^3) and wrapped *E. sibiricus* silage (four randomly selected bales; density >700 kg/m^3), respectively, on September 5, 2019. The wrapped silages were ensiled for about 350 days on an experimental farm of Inner Mongolia Academy of Agriculture and Animal Husbandry Science, Hohhot, China. The samples were separately packed in a plastic bag (food grade, 300 mm $\times$ 400 mm; Qingye, Beijing, China) by a vacuum sealer (DZ-300; Qingye, Beijing, China), and then transferred to the laboratory in an ice box. The silages (20 g) were individually mixed uniformly with 180 ml of sterile Man, Rogosa, Sharpe (MRS) broth in a reagent bottle (200 ml) by a shaker (HY-150, Wuhan Huicheng Biological Technology Co., Ltd., Wuhan, China) at 4°C for 30 min and then cultured

at 37°C in an incubator (LRH-70, Shanghai Yiheng Scientific Instruments Co., Ltd., Shanghai, China) for 24 h. The 20 ml of LAB suspensions was drawn from each reagent bottle, placed into a sterilized centrifuge tube, and then stored at −80°C for analyzing bacterial community. The pH and LAB count of the silage samples and the LAB suspensions were analyzed. The LAB suspensions were stored at 4°C and used as an inoculant for ensiling whole-plant corn in 5 days.

Preparing Silages and Sampling

Corn (*Zea mays* L.) was grown on the same farm. Whole corn plants were harvested at the two-thirds milk-line stage from four different fields as replicates on September 10, 2019. The fresh forages from each field were separately chopped into 1- to 2-cm pieces, mixed thoroughly, and then divided into three batches of film for three treatments as follows: CK, spraying 3.0 ml/kg fresh weight (FW) of distilled water; CSL, spraying 3.0 ml/kg FW of LAB suspension made from whole-plant corn silage (LAB suspension-CS); and ESL, spraying with a mixture of 1.0 ml/kg FW of LAB suspension made from *E. sibiricus* silage (LAB suspension-ES) and 2.0 ml/kg (FW) distilled water. After mixing uniformly, approximately 500 g of forage was packed into a plastic bag and sealed with a vacuum sealer; 44 bags of silage were prepared per treatment (11 bags per field), and a portable oxygen meter (KP810, Henan Zhong an Electronic Detection Technology Co., Ltd., Zhengzhou, China) was sealed in a bag per treatment of each field for detecting oxygen content. The silages were stored in laboratory and sampled after ensiling for 0 h, anaerobic state (A), 3 h, 5 h, 10 h, 24 h, 2 days, 3 days, 10 days, 30 days, and 60 days. When the oxygen content in silages reduced to 0, the silage was in anaerobic state. The 20 g of sample from each bag was placed into a self-styled bag and stored at −80°C for analyzing bacterial community.

Analysis

The silages were dried in a forced-air oven (BPG-9240A, Shanghai Yiheng Scientific Instrument Co., Ltd., Shanghai, China) at 65°C for 48 h, ground through a 1-mm screen using a mill (FS-6D; Fichi Machinery Equipment Co., Ltd., Shandong, China) and then dried at 105°C until reaching a constant mass for detecting dry matter content. The mixture of 25 g fresh silage and 225 ml sterile water was homogenized for 100 s in a flap-type sterile homogenizer (JX-05, Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China) and filtered through four layers of cheesecloth to prepare silage extracts. The pH of silage was measured by pH meter; the counts of LAB, Coliforms, bacteria, and yeast were determined by culturing on MRS agar, violet red bile agar, nutrient agar, and potato dextrose agar, respectively, at 37°C for 48 h (Cai, 1999).

The bacterial community of the LAB suspensions and the whole-plant corn silages were analyzed. The DNA extraction was operated using the E.Z.N.A. ®Stool DNA Kit (D4015, Omega, Inc., United States) according to the manufacturer's instructions. The V3–V4 region of the bacterial rRNA gene was amplified by polymerase chain reaction (PCR) (98°C for 30 s followed by 32 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s and a final extension at 72°C for 10 min) with primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Logue et al., 2016). The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, United States) and quantified by Qubit (Invitrogen, United States). The samples were sequenced on an Illumina NovaSeq PE250 platform according to the manufacturer's recommendations. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH. Quality filtering on the raw reads was performed under specific filtering conditions to obtain the high-quality clean tags according to the fqtrim (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). After dereplication using DADA2, the feature table and feature sequence were obtained. Alpha diversity and beta diversity were calculated by QIIME2, the sequence alignment of species annotation was performed by BLAST, and the alignment database was SILVA and NT-16S. The sequencing data were submitted to the NCBI Sequence Read Archive database (accession number: PRJNA693042). The stacked bars of bacterial genera were made by Excel (Microsoft 365, Microsoft Corporation, Seattle, WA, United States) according to the relative abundance of the bacterial community. The difference in the bacterial community among treatments or ensiling time was analyzed using the Mann–Whitney *U* test and the Kruskal–Wallis test by R version 3.6.1.

Statistical Analyses

The LAB Count and pH of LAB suspension whole-plant corn silage, and *E. sibiricus* silage were analyzed using GLIM procedure of SAS (version 9.1.3; SAS Inst. Inc., Cary, NC, United States). For the ensiling experiments, data on dry matter, pH, and microbial counts of whole-plant corn silage were analyzed as 3×2 factorial design, 3×7 factorial design, and 3×4 factorial design during the initial aerobic phase, the intense fermentation phase, and the stable phase, respectively. The model included effects of additives, storage time, and their interaction. Differences among additives, and among storage times, were analyzed with the GLIM procedure of SAS. The interaction of additives and storage time was analyzed using the PDIFF procedure of SAS. The correlation between bacterial genera and pH were performed by R version 3.6.1 using the OmicStudio tools at https://www.omicstudio.cn/tool.

RESULTS

Characteristic of LAB Suspension

Whole-plant corn silages for preparing LAB suspension had lower pH and less LAB count than *E. sibiricus* silages ($P < 0.05$). Compared to LAB suspension-ES, the LAB suspension-CS had higher pH and lower LAB count ($P < 0.05$) (**Table 1**).

The dominant genera in LAB suspension-CS were *Lactobacillus*, *Weissella*, *Lachnoclostridium*_5, and *Clostridium*_sensu_stricto_12 with abundances of 40.3, 37.6, 16.6, and 5.01%, respectively. However, *Lactobacillus* was the most predominant genus in LAB suspension-ES (96.4%), followed by *Pediococcus* and *Clostridium*_sensu_stricto_12

TABLE 1 | pH and lactic acid bacteria (LAB) counts of whole-plant corn silage and *Elymus sibiricus* silage (log_{10} colony-forming units/g fresh weight), and of LAB suspension (log_{10} colony-forming units/ml) prepared from the two silages.

Items		Whole-plant corn silage	*Elymus sibiricus* silage	SEM	*P* value
Silages	pH	3.51b	4.71a	0.0128	<0.0001
	LAB	6.05b	7.59a	0.0398	<0.0001
LAB suspension	pH	4.55a	3.76b	0.0176	<0.0001
	LAB	8.49b	9.02a	0.0251	<0.0001

Values with different lowercase letters show significant differences ($P < 0.05$). SEM, standard error of means.

with abundances of 2.07 and 0.50%, respectively (**Figure 1A**). Additionally, LAB suspension-CS contained greater *Weissella*, *Lachnoclostridium*_5, *Clostridium*_sensu_stricto_12, and *Staphylococcus*, and lower *Lactobacillus* and *Pediococcus* than LAB suspension-ES ($P < 0.05$) (**Figure 1B**).

Initial Aerobic Phase

The CSL_0 h and ESL_0 h had lower pH and greater counts of LAB and coliforms than CK_0 h ($P < 0.05$); additionally, ESL_A contained lower pH and higher coliform count than CK_A ($P < 0.05$). The CK_A had higher pH and LAB count than CK_0 h ($P < 0.05$), and CSL_A had greater pH than CSL_0 h ($P < 0.05$) (**Table 2**). The initial aerobic phase time of CSL was longer than that of CK and ESL ($P < 0.05$) (**Figure 2**). There was no interactive effect detected between storage time and additives.

During this phase, the predominant bacterial genera in CK, CSL, and ESL were *Pantoea*, *Pseudomonas*, *Sphingomonas*, unclassified *Gammaproteobacteria*, *Klebsiella*, *Rahnella*, *Enterobacter*, and *Chryseobacterium* with more than 1% of

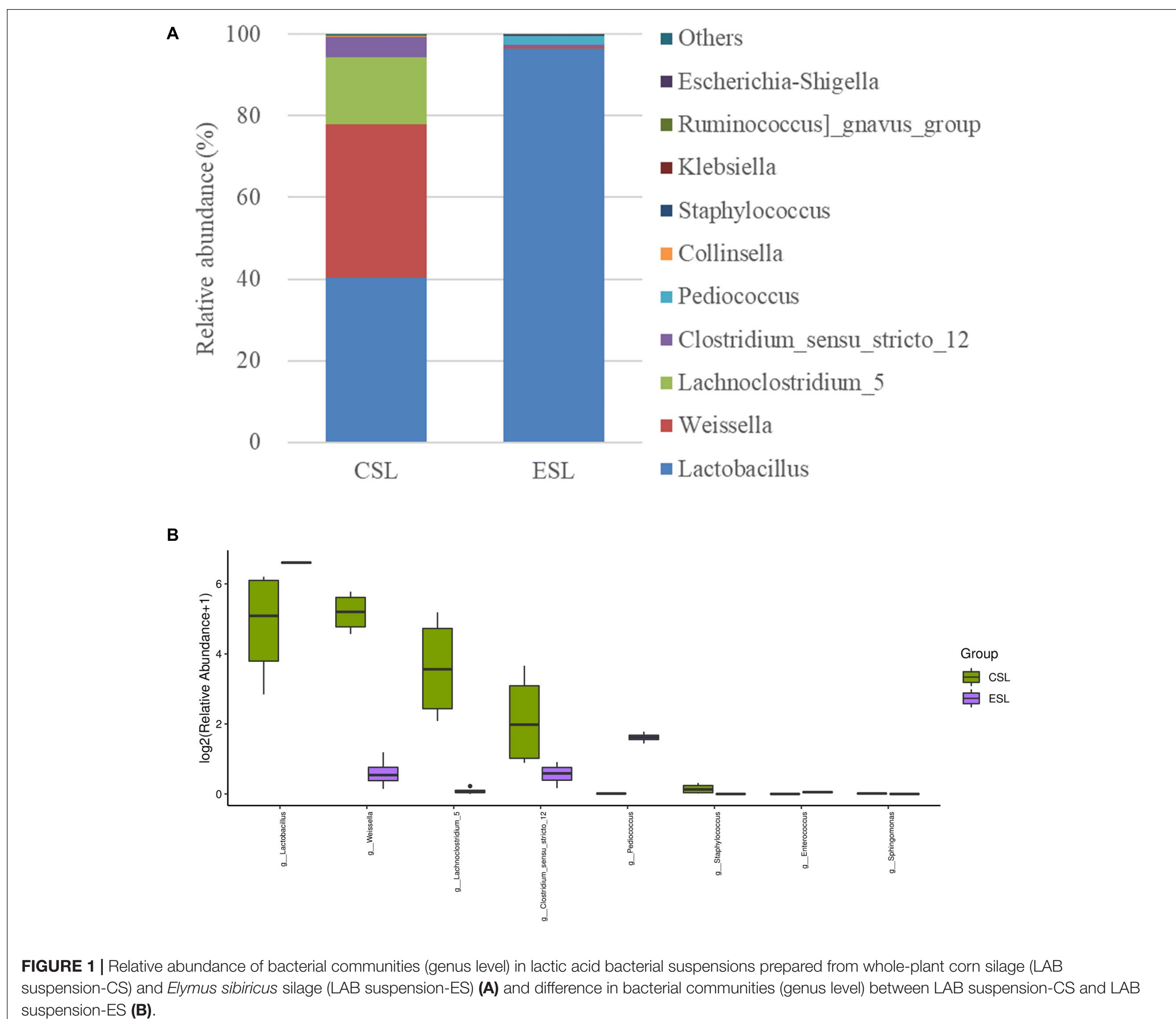

FIGURE 1 | Relative abundance of bacterial communities (genus level) in lactic acid bacterial suspensions prepared from whole-plant corn silage (LAB suspension-CS) and *Elymus sibiricus* silage (LAB suspension-ES) **(A)** and difference in bacterial communities (genus level) between LAB suspension-CS and LAB suspension-ES **(B)**.

TABLE 2 | Dry matter (DM, g/kg) and microbial counts (log_{10} colony-forming units/g fresh weight) of whole-plant corn silage during the initial aerobic phase.

Items		CK	CSL	ESL	SEM	*P* value
DM	0 h	389	364	378	6.2533	0.0612
	A	385	369	372	5.1200	0.1141
	SEM	6.4928	6.8648	2.9492		
	P value	0.6972	0.6593	0.1847		
pH	0 h	6.05Ab	5.88Bb	5.89B	0.0263	0.0022
	A	6.14Aa	6.03ABa	5.94B	0.0340	0.0080
	SEM	0.0173	0.0291	0.0404		
	P value	0.0150	0.0115	0.4851		
Lactic acid bacteria	0 h	6.19bB	6.83A	6.93A	0.1939	0.0473
	A	6.59a	6.68	6.55	0.1023	0.6735
	SEM	0.0988	0.0837	0.2353		
	P value	0.0267	0.2385	0.2884		
Yeasts	0 h	6.73	7.04	7.15	0.2096	0.3818
	A	6.90	7.05	6.90	0.1098	0.5425
	SEM	0.1461	0.1590	0.1933		
	P value	0.4295	0.9575	0.3957		
Coliforms	0 h	7.20B	7.53A	7.47A	0.0669	0.0174
	A	7.22B	7.42AB	7.53A	0.0751	0.0504
	SEM	0.0763	0.0518	0.0816		
	P value	0.8766	0.1926	0.6219		
Bacteria	0 h	7.56	8.32	7.88	0.2032	0.0770
	A	7.61	8.28	7.86	0.2377	0.1853
	SEM	0.0454	0.3770	0.0499		
	P value	0.5098	0.9498	0.7605		

Values with different lowercase letters (a and b) indicate significant differences among treatments on the same time. Values with different uppercase letters (A and B) indicate significant differences between 0 h and anaerobic state (A). CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from Elymus sibiricus silage. A, anaerobic state. SEM, standard error of means.

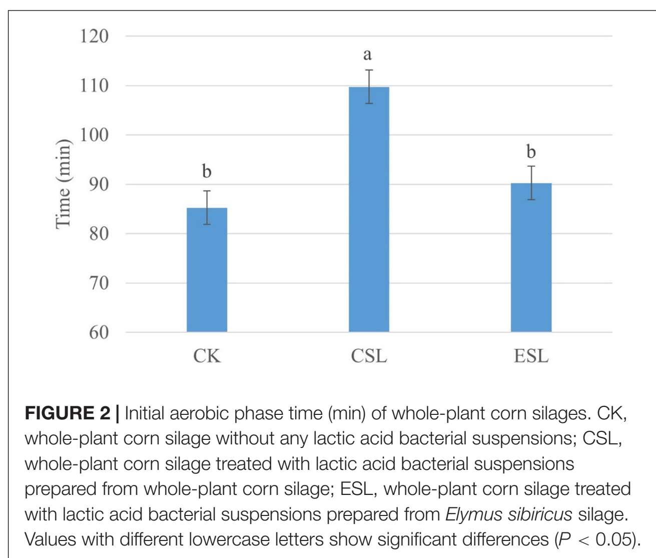

FIGURE 2 | Initial aerobic phase time (min) of whole-plant corn silages. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage. Values with different lowercase letters show significant differences ($P < 0.05$).

abundance (**Figure 3**). CK_A contained greater *Klebsiella*, *Enterobacter*, *Rahnella*, unclassified *Gammaproteobacteria*, *Serratia*, and *Erwinia*, and lower *Sphingomonas* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* than CK_0 h ($P < 0.05$). CSL_A contained higher *Rahnella*, *Rosenbergiella*, *Klebsiella*, and *Erwinia*, and lower *Sphingomonas*, *Chryseobacterium*, *Hymenobacter*, *Asaia*, *Stenotrophomonas*, unclassified *Mitochondria*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Massilia*, and *Curtobacterium* than CSL_0 h ($P < 0.05$); ESL_A contained higher *Serratia* and lower *Stenotrophomonas* and *Curtobacterium* than ESL_0 h ($P < 0.05$) (**Figure 4**). The abundance of *Weissella* in CK_A and ESL_A was greater than in CK_0 h and ESL_0 h, respectively ($P < 0.05$); however, *Lactobacillus* and *Pediococcus* decreased in CSL during this phase ($P < 0.05$) (**Figure 4**).

Intense Fermentation Phase

During this phase, the pH and the coliform count decreased ($P < 0.05$), while the counts of LAB, bacteria, and yeast increased ($P < 0.05$). The ESL had lower pH at A and 10 h, and higher pH at 3 h than CK and CSL ($P < 0.05$). Comparing with CK, the CSL and ESL had lower pH after 24 h ($P < 0.05$), and higher LAB count from 3 h to 2 days ($P < 0.05$). The storage time impacted pH and counts of LAB, coliforms, bacteria, and yeast ($P < 0.05$); the additives (LAB suspension) affected pH and counts of LAB, bacteria, and yeast ($P < 0.05$), which were also interactively influenced by storage time and additives ($P < 0.05$) (**Table 3**).

During this phase, the abundance of *Lactobacillus* in CK and CSL increased to 70.5 and 84.5% at 3 days, respectively; however, *Weissella* increased to 42.4 and 33.8% at 10 h and then decreased to 4.05 and 2.10% at 3 days, respectively. Additionally, *Lactococcus* and *Leuconostoc* in CK and CSL went up in the first 24 h and then turned down. *Pediococcus* in CK increased to 3.94% at 2 days and then decreased to 2.28% at 3 days. *Pediococcus* in CSL increased to 2.85% at 24 h and then reduced to 1.34% at 3 days. In the ESL, *Lactobacillus* increased to 62.3% at 2 days and then decreased to 49.9% at 3 days; moreover, *Weissella*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* went up in the first 24 h and down at 2 days, and then went up a little bit at 3 days. *Pantoea* in CK and ESL went down during this phage, while in CSL, it increased to 37.0% at 3 h and then went down to 0.67% at 3 days. Moreover, *Klebsiella* and *Enterobacter* in CK, CSL, and ESL went up in the first 5 h and then turned down (**Figure 5**).

Stable Phase

During this phase, the pH and the counts of LAB, yeast, coliforms, and bacteria decreased in CK, CSL, and ESL ($P < 0.05$). The CSL and ESL had lower pH than CK, and ESL contained more yeast count than CK and CSL ($P < 0.05$). The storage time and the additives had an effect on pH ($P < 0.05$) (**Table 4**).

During this phase, the abundance of *Lactobacillus* reached 80.5% in CK and 85.1% in CSL at 10 days, and 80.1% in ESL at 30 days, and then reduced to 57.9, 61.8, and 48.7% at 60 days, respectively. The opposite trends were observed in *Weissella* (reduced to 1.81 and 1.26% in CK and CSL at 10 days and 2.19% in ESL at 30 days, respectively) and *Leuconostoc* (reduced to 0.83 and 0.66% in CK and CSL at 10 days and 0.86% in ESL at 30 days, respectively). *Lactococcus* in CK, CSL, and ES decreased to 0.85, 0.51, and 0.52% at 60 days, respectively. *Pediococcus* went down to 1.57% in CK and to 0.49% in ESL at 30 days and then up to 2.04

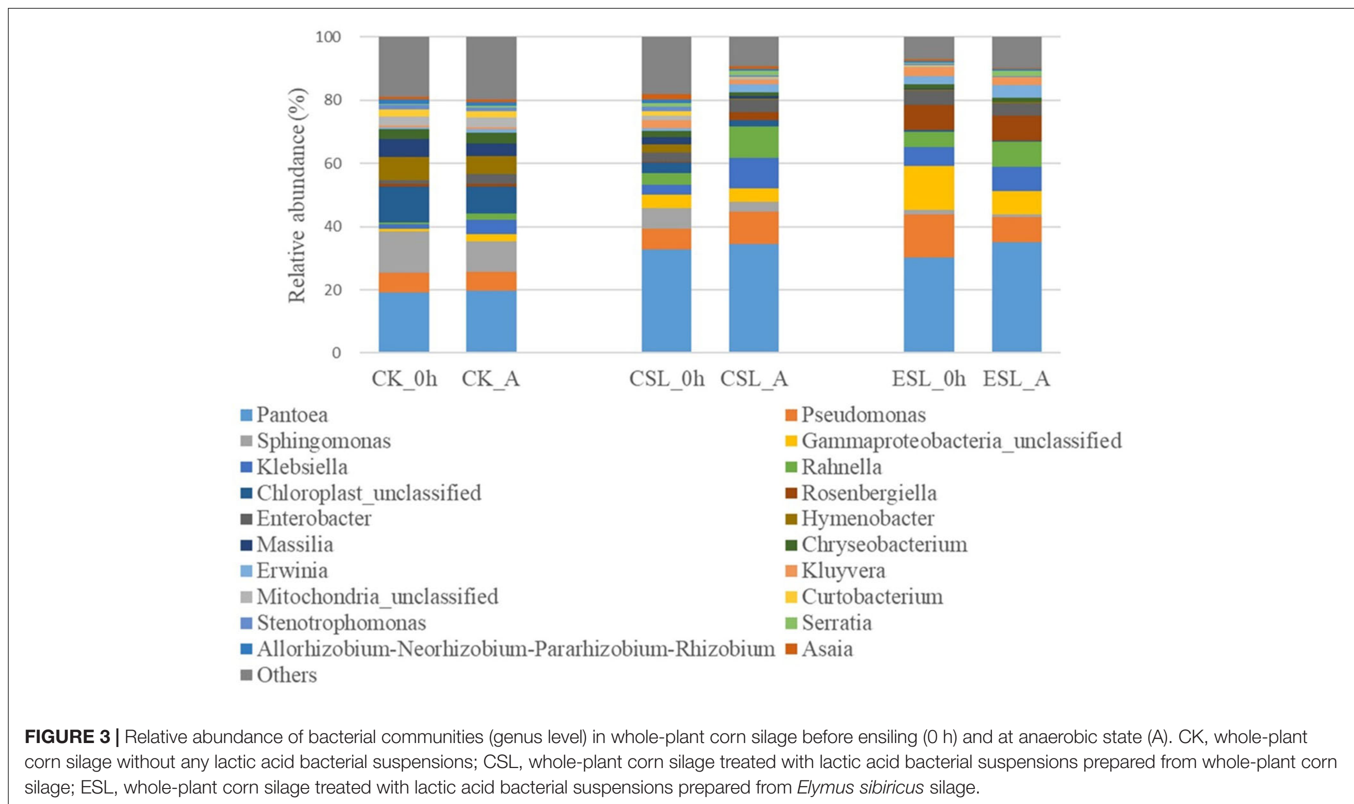

FIGURE 3 | Relative abundance of bacterial communities (genus level) in whole-plant corn silage before ensiling (0 h) and at anaerobic state (A). CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage.

and 0.92% at 60 days, respectively, and that in CSL decreased to 0.57% at 60 days (**Figure 6**).

Correlations Between Main Bacterial Genera and pH

From 0 to 5 h, the most dominant bacterial genus was *Pantoea* in silages with LAB population as minor taxa (**Figures 3**, **5**). The pH had negative correlations with *Weissella*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Lactobacillus*, *Klebsiella*, and *Enterobacter* ($P < 0.05$), and positive correlation with *Pseudomonas* ($P < 0.05$). *Klebsiella* correlated negatively with *Pseudomonas* ($P < 0.05$) and positively with *Weissella* and *Enterobacter* ($P < 0.05$). *Pseudomonas* correlated positively with *Pantoea* and unclassified *Gammaproteobacteria* ($P < 0.05$) and had negative correlation with *Weissella* ($P < 0.05$) (**Figures 7A,B**).

The most dominant bacterial genus was *Weissella* from 5 to 24 h in silages with LAB population increasing rapidly (**Figure 5**). The pH had negative correlations with *Weissella*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* ($P < 0.05$), and positive correlations with *Klebsiella*, *Pantoea*, *Enterobacter*, and *Sphingomonas* ($P < 0.05$). *Weissella* correlated positively with *Lactococcus* and *Leuconostoc* ($P < 0.05$) and negatively with *Klebsiella*, *Pantoea*, *Enterobacter*, and *Sphingomonas* ($P < 0.05$) (**Figure 7C**).

Lactobacillus dominated the bacterial community from 24 h to 60 days (**Figure 6**). The pH had negative correlations with *Lactobacillus* and *Sphingomonas* and positive correlations with *Weissella*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Rahnella* ($P < 0.05$). *Lactobacillus* correlated negatively with *Weissella*, *Klebsiella*, *Lactococcus*, *Pantoea*, *Leuconostoc*, *Enterobacter*, and *Pediococcus* ($P < 0.05$) (**Figure 7C**).

DISCUSSION

Whole-plant corn silages for preparing LAB suspension had lower pH and less LAB count than *E. sibiricus* silages; moreover, LAB suspension-CS contained higher pH and lower LAB count than LAB suspension-ES (**Table 1**). Those suggested that the LAB in LAB suspension-CS might have good acid tolerance, but the poor capacity of producing acid and proliferation. *Lactobacillus* was the most predominant bacterial genus in LAB suspension-CS and LAB suspension-ES (**Figure 1**). Additionally, LAB suspension-CS contained greater abundances of *Weissella*, *Lachnoclostridium*_5, and *Clostridium*_sensu_stricto_12 than ESL, which might be due to their inhibited activity by lower pH in LAB suspension-ES (**Table 1**). *Lachnoclostridium*_5 and *Clostridium*_sensu_stricto_12 were also detected in whole-plant corn silage (Keshri et al., 2018) and in mulberry leave silage and stylo silage (He et al., 2020). The optimum condition for *Lachnoclostridium* growth is neutral to alkaline pH and 20–63°C, and the main fermentation product is acetate by fermenting mono- and disaccharides (Yutin and Galperin, 2013). The high moisture and pH (>4.0) in LAB suspension-CS might provide the satisfactory growing condition for *Clostridium* that is an undesirable

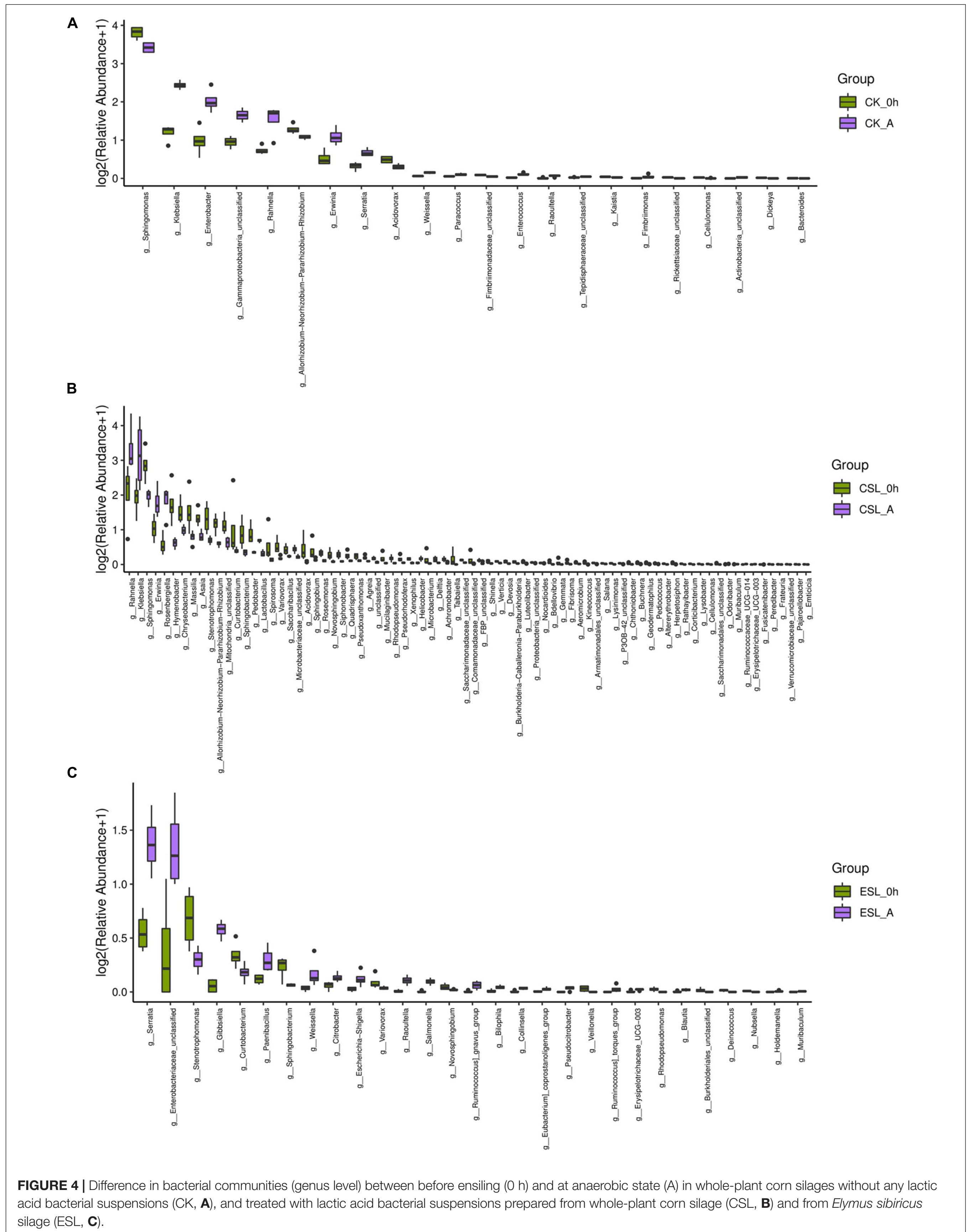

FIGURE 4 | Difference in bacterial communities (genus level) between before ensiling (0 h) and at anaerobic state (A) in whole-plant corn silages without any lactic acid bacterial suspensions (CK, **A**), and treated with lactic acid bacterial suspensions prepared from whole-plant corn silage (CSL, **B**) and from *Elymus sibiricus* silage (ESL, **C**).

TABLE 3 | Dry matter (DM, g/kg) and microbial counts ($\log_{10}$ colony-forming units/g fresh weight) of whole-plant corn silage during the intense fermentation phase.

Items		CK	CSL	ESL	SEM	*P* value	Interaction		
							T	A	T × A
DM	A	385	369ab	372b	5.1200	0.1141		***	*
	3 h	386	375a	379ab	6.1616	0.4881			
	5 h	392A	359abB	377abA	4.8634	0.0035			
	10 h	388A	348bB	380abA	3.3344	<0.0001			
	24 h	391A	365abB	391aA	4.6015	0.0048			
	2 days	379	374a	375ab	3.5375	0.5541			
	3 days	382	371ab	379ab	4.6533	0.2643			
	SEM	4.2870	5.8195	3.7257					
	P value	0.3695	0.0420	0.0573					
pH	A	6.14aA	6.03aAB	5.94bB	0.0340	0.0080	***	***	***
	3 h	5.95bB	5.94bB	6.02aA	0.0129	0.0036			
	5 h	5.58c	5.63c	5.53c	0.0235	0.0550			
	10 h	5.11dA	5.10dA	4.89dB	0.0463	0.0135			
	24 h	4.63eA	4.53eB	4.58eAB	0.0178	0.0137			
	2 d	4.25fA	4.04fC	4.14fB	0.0090	<0.0001			
	3 d	4.01gA	3.83gB	3.86gB	0.0109	<0.0001			
	SEM	0.0243	0.0275	0.0244					
	P value	<0.0001	<0.0001	<0.0001					
Lactic acid bacteria	A	6.59d	6.68d	6.55d	0.1023	0.6735	***	***	***
	3 h	6.78dB	7.24cA	7.30cA	0.1039	0.0123			
	5 h	8.35cB	8.76bA	9.00bA	0.0924	0.0024			
	10 h	8.70bB	9.37aA	9.41aA	0.0530	<0.0001			
	24 h	9.24aB	9.44aA	9.57aA	0.0488	0.0028			
	2 days	9.26aB	9.41aA	9.44aA	0.0454	0.0448			
	3 days	9.26a	9.52a	9.46a	0.0700	0.0597			
	SEM	0.0744	0.0797	0.0780					
	P value	<0.0001	<0.0001	<0.0001					
Yeast	A	6.90f	7.05d	6.90d	0.1098	0.5425	***	***	***
	3 h	6.84f	7.00d	6.78d	0.0920	0.2630			
	5 h	7.17eB	7.77cA	7.38cB	0.0996	0.0065			
	10 h	7.63dB	8.24bA	8.15bA	0.0734	0.0005			
	24 h	8.10cC	8.27bB	8.42aA	0.0444	0.0022			
	2 days	8.36bB	8.42bA	8.43aA	0.0120	0.0053			
	3 days	8.62aC	8.78aA	8.68aB	0.0097	<0.0001			
	SEM	0.0776	0.0659	0.0770					
	P value	<0.0001	<0.0001	<0.0001					
Coliforms	A	7.22a	7.42a	7.53a	0.0751	0.0504	***		
	3 h	7.54a	7.37a	7.64a	0.1017	0.2256			
	5 h	7.39a	7.49a	7.61a	0.0775	0.1830			
	10 h	7.71a	7.73a	7.87a	0.0628	0.1986			
	24 h	7.65aA	7.42aB	7.69aA	0.0603	0.0223			
	2 days	5.77bA	4.31bB	5.79bA	0.0954	<0.0001			
	3 days	3.23c	3.37b	2.67c	0.7648	0.7929			
	SEM	0.1781	0.4451	0.1935					
	P value	<0.0001	<0.0001	<0.0001					
Bacteria	A	7.61f	8.28c	7.86e	0.2377	0.1853	***	***	***
	3 h	7.70ef	7.80d	7.84e	0.0526	0.2051			
	5 h	7.88eB	8.70bcA	8.77dA	0.0614	<0.0001			
	10 h	8.27dB	8.91abA	8.97cA	0.0285	<0.0001			
	24 h	8.94c	9.14ab	9.21b	0.0920	0.1559			
	2 days	9.19bB	9.40aA	9.47aA	0.0524	0.0112			
	3 days	9.39a	9.45a	9.51a	0.0796	0.5857			
	SEM	0.0639	0.1621	0.0673					
	P value	<0.0001	<0.0001	<0.0001					

*Values with different lowercase letters (a, b,... g) indicate significant differences among anaerobic state, 3 h, 5 h, 10 h, 24 h, 2 days, and 3 days after ensiling of each treatment. Values with different uppercase letters (A, B, and C) indicate significant differences among treatments on the same time during the intense fermentation phase. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from Elymus sibiricus silage. A, anaerobic state. SEM, standard error of means. *, $P < 0.05$; ***, $P < 0.001$.*

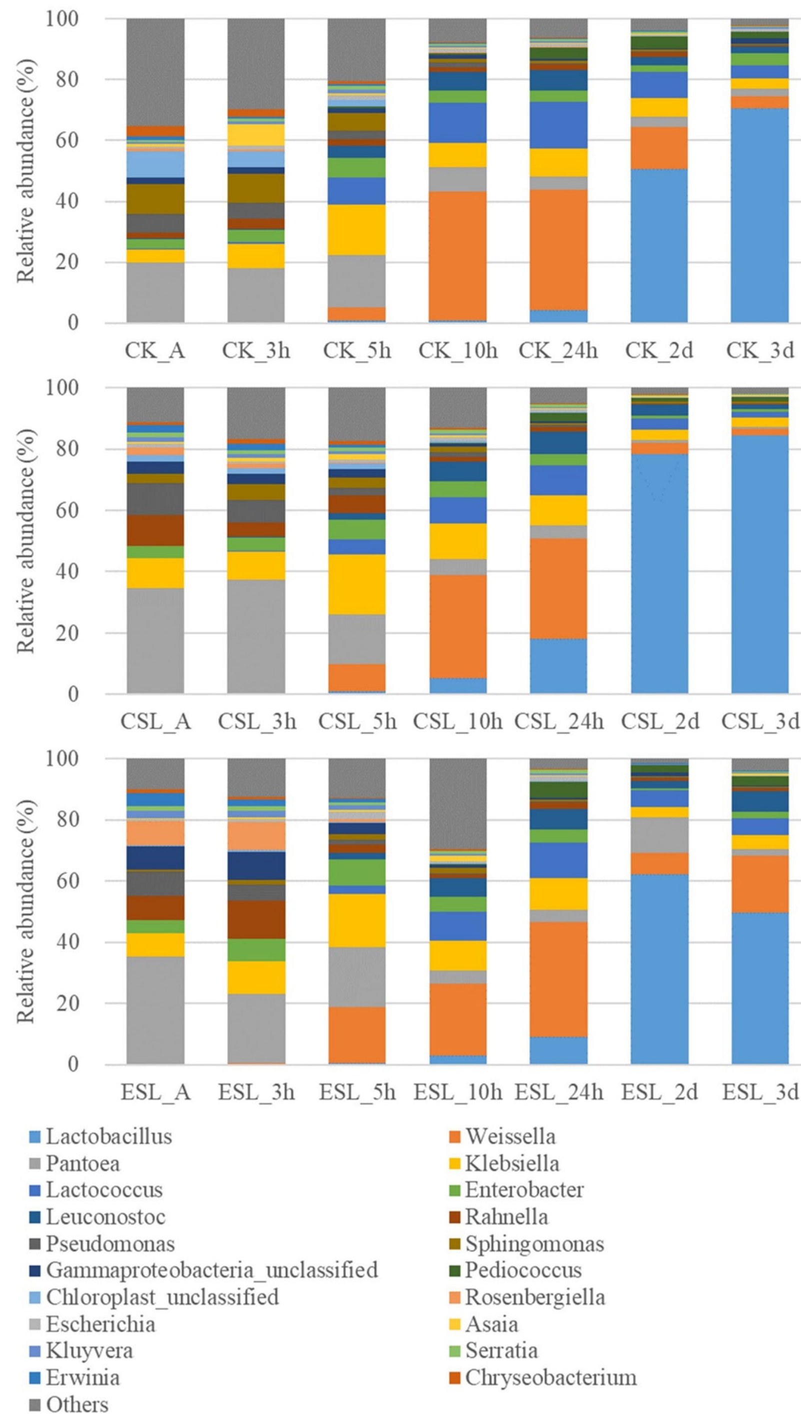

FIGURE 5 | Relative abundance of bacterial communities (genus level) in whole-plant corn silages during the intense fermentation phase. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage; A, anaerobic state.

microorganism that endangered the fermentation quality of silage (Carvalho-Estrada et al., 2020).

The ensiling process was divided into the initial aerobic phase, the intense fermentation phase, the stable phase, and the aerobic feed-out to better understand the main reactions in silage (Weinberg and Muck, 1996). Ávila and Carvalho (2019) reported the main microorganisms in each phase of fermentation process. In the present study, the initial aerobic phase, the intense

TABLE 4 | Dry matter (DM, g/kg) and microbial counts ($\log_{10}$ colony-forming units/g fresh weight) of whole-plant corn silage during the stable phase.

Items		CK	CSL	ESL	SEM	*P* value	Interaction		
							T	A	T × A
DM	3 days	382ab	371	379	4.6533	0.2643		**	**
	10 days	373b	379	381	3.4621	0.3005			
	30 days	387a	378	380	2.8565	0.0992			
	60 days	388aA	369B	389A	3.9423	0.0106			
	SEM	3.3796	4.0575	3.8884					
	P value	0.0354	0.2538	0.2715					
pH	3 days	4.01aA	3.83bB	3.86aB	0.0107	<0.0001	***	***	***
	10 days	3.85b	3.82b	3.83a	0.0082	0.0550			
	30 days	3.90bA	3.87aB	3.86aB	0.0067	0.0136			
	60 days	3.65c	3.58c	3.58b	0.0206	0.0725			
	SEM	0.0149	0.0103	0.0128					
	P value	<0.0001	<0.0001	<0.0001					
Lactic acid bacteria	3 days	9.26a	9.52a	9.46a	0.0699	0.0597	***		
	10 days	8.71b	8.99b	8.94b	0.1188	0.2561			
	30 days	8.30c	8.17c	8.20c	0.0675	0.4381			
	60 days	7.68d	7.64d	7.70d	0.0321	0.4029			
	SEM	0.1040	0.0463	0.0741					
	P value	<0.0001	<0.0001	<0.0001					
Yeast	3 days	8.62aC	8.78aA	8.68aB	0.0097	<0.0001	***		
	10 days	8.33bB	8.37bB	8.47aA	0.0173	0.0007			
	30 days	8.03c	7.95c	7.99b	0.1116	0.8877			
	60 days	7.23d	7.21d	7.19c	0.0982	0.9544			
	SEM	0.0601	0.0799	0.0829					
	P value	<0.0001	<0.0001	<0.0001					
Coliforms	3 days	3.23a	3.37a	2.67a	0.7648	0.7929	***		
	10 days	ND	ND	ND	–	–			
	30 days	ND	ND	ND	–	–			
	60 days	ND	ND	ND	–	–			
	SEM	0.2101	0.5817	0.2369					
	P value	<0.0001	0.0028	<0.0001					
Bacteria	3 days	9.39a	9.45a	9.51a	0.0796	0.5857	***		
	10 days	8.71b	8.85b	8.64b	0.1030	0.3807			
	30 days	8.12c	8.14c	8.18c	0.0729	0.8331			
	60 days	7.19d	6.91d	7.25d	0.1089	0.1248			
	SEM	0.0969	0.0876	0.0923					
	P value	<0.0001	<0.0001	<0.0001					

*Values with different lowercase letters (a, b, c, and d) indicate significant differences among 3, 10, 30, and 60 days after ensiling of each treatment. Values with different uppercase letters (A, B, and C) indicate significant differences among treatments on the same time during the stable phase. ND, no detected. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from Elymus sibiricus silage. SEM, standard error of means. **, $P < 0.01$; ***, $P < 0.001$.*

fermentation phase, and the stable phase were determined by the oxygen content, the increasing LAB count (pH >4.0), and the decreasing LAB count (pH <4.0), respectively.

The LAB suspensions were cultured in MRS broth anaerobically; however, the LAB was suddenly exposed to oxygen after spraying on ensiling materials. Because of the decreased oxygen-scavenging ability, the complete absence of the oxygen-scavenging system, as well as the accumulation of toxic oxygen metabolites (Zhang and Cai, 2014), the LAB counts in CSL and ESL decreased during the initial aerobic phase (**Table 2**). Nevertheless, the original epiphytic LAB on whole-plant corn might have the greater oxygen-scavenging ability, owing to the constant exposure to air before ensiling, which resulted in an increasing LAB count in CK during this phase (**Table 2**). The respiration of aerobic microorganisms and plant cells, and the fermentation of facultatively aerobic or anaerobic bacteria occur synchronously in silage during the initial aerobic phase (Ávila and Carvalho, 2019); those cooperating with plant and microbial proteolytic enzymes cause proteolysis and increase of the buffer capacity in silage (Tao et al., 2012). In the present study, *Pseudomonas* presented in silage with considerable abundance (**Figure 3**) and can produce proteolytic enzymes and result in

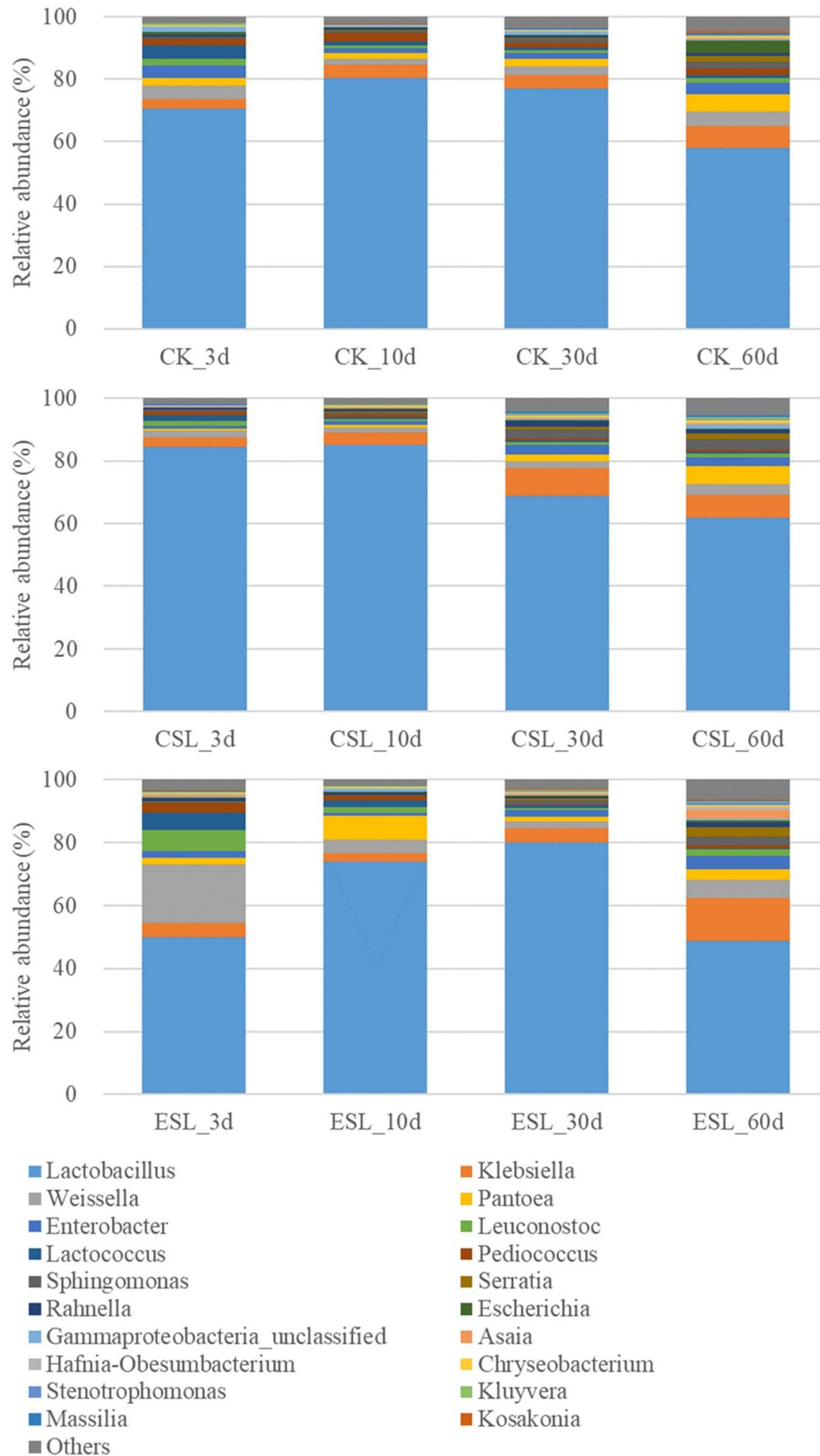

FIGURE 6 | Relative abundance of bacterial communities (genus level) in whole-plant corn silages during the stable phase. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage.

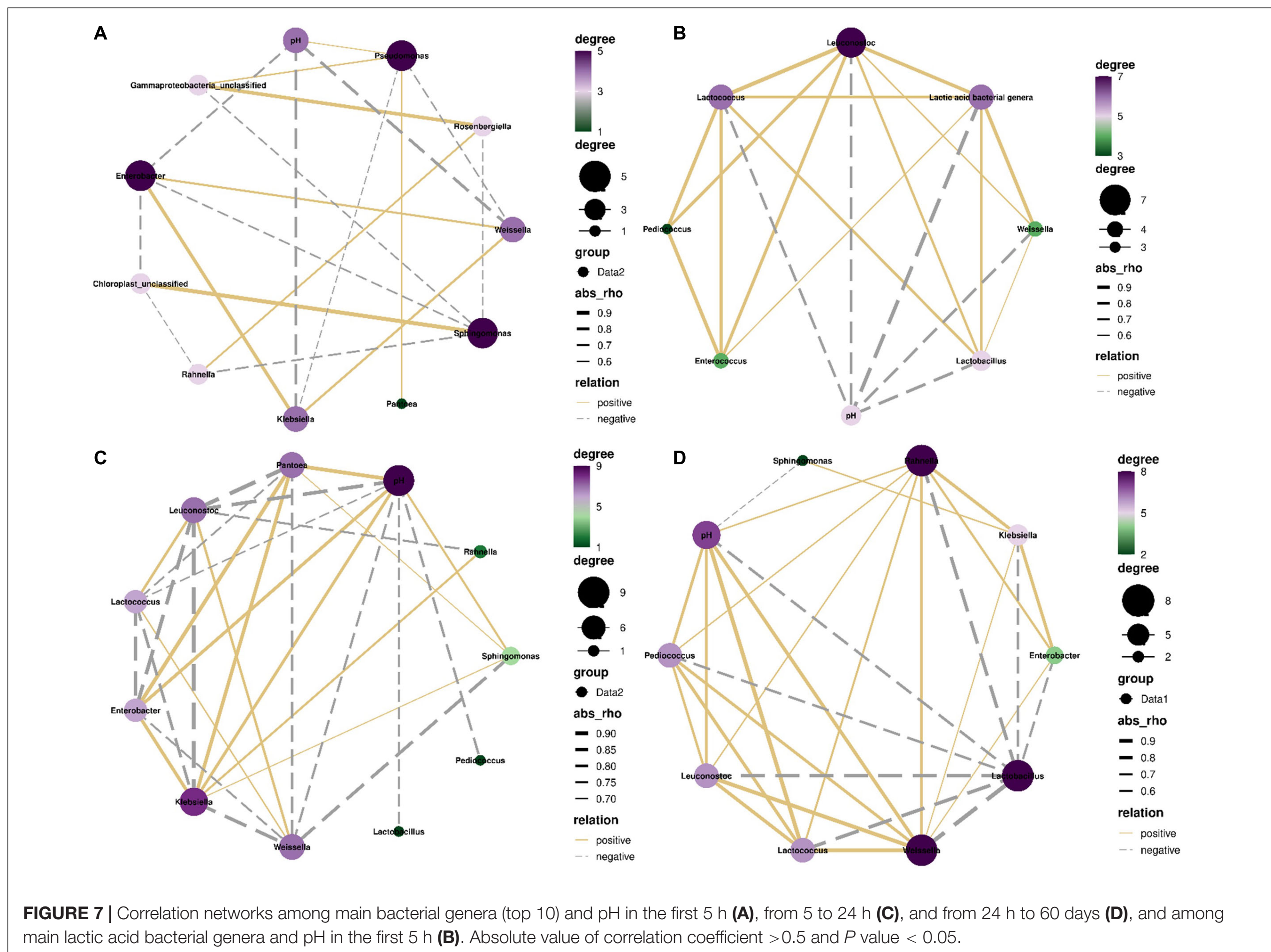

FIGURE 7 | Correlation networks among main bacterial genera (top 10) and pH in the first 5 h **(A)**, from 5 to 24 h **(C)**, and from 24 h to 60 days **(D)**, and among main lactic acid bacterial genera and pH in the first 5 h **(B)**. Absolute value of correlation coefficient >0.5 and *P* value < 0.05.

protein degradation (Scatamburlo et al., 2015; Paludetti et al., 2020). Those might result in an increase of pH during the initial aerobic phase (**Table 2**). Additionally, the respiration is much greater than the fermentation and the initial aerobic phase might be mainly depending on the aerobic respiration in silages (Ávila and Carvalho, 2019). In the current study, the duration of the initial aerobic phase was 85, 109, and 90 min in CK, CSL, and ESL, respectively (**Figure 2**), with 34.6, 25.2, and 0.95% of aerobic bacteria, and 28.2, 57.6, and 86.2% of facultatively anaerobic bacteria in materials, respectively (**Supplementary Figure 1**). The effect of the respiration of plants and microbes on the initial aerobic phase needs further research. After ensiling, the initial microbial activity is mostly aerobic microflora and then inhibited by an anaerobic or/and sufficiently acidic environment in silage (Dunière et al., 2013). In this study, during the initial aerobic phase, the abundances of *Pantoea*, *Klebsiella*, *Rahnella*, *Erwinia*, and *Serratia* increased in CK, CSL, and ESL, as well as *Enterobacter* in CK and CSL (**Figures 3**, **4**). Nevertheless, the LAB population (*Weissella*, *Enterococcus*, *Lactobacillus*, and *Pediococcus*) present in the silages as minor taxa (**Figure 3** and **Supplementary Figure 2A**), and the bacterial counts increased in CK and decreased in CSL and ESL (**Table 2**). Those implied that *Enterobacteriaceae* and *Erwiniaceae* represented the major aerobic bacterial activity and LABs have weak activity in whole-plant corn silages during the initial aerobic phase.

During the intense fermentation phase, the most dominant bacterial genus was *Pantoea* from A to 3 h or 5 h with pH >5.5, *Weissella* from 10 to 24 h with pH >4.50 and <5.2, and *Lactobacillus* after 2 days with pH ≤4.25, respectively (**Figure 5** and **Table 3**). Those implied that two shifts of the most predominant bacterial genus (from *Pantoea* to *Weissella* and then to *Lactobacillus*) occurred in whole-plant corn silages during this phase. Similarly, Keshri et al. (2018) found that the most dominant bacterial genus in whole-plant corn silages was *Acinetobacter* in the first 3 h, *Weissella* from 5 h to 1 day, and then *Lactobacillus* after ensiling for 2 days. The LAB count increased exponentially from A to 24 h (**Table 3**); the abundance of *Weissella*, *Lactococcus*, and *Leuconostoc* had increased before 10 h in CK, CSL, and ESL, while the abundance of Lactobacillus in CK reduced in the first 10 h while it decreased in CSL and ESL in the first 5 h after that they increased (**Supplementary Figure 3**). Moreover, the CSL and ESL contained greater *Lactobacillus* than CK at 10 h, 24 h, and 2 days (**Supplementary Figure 2**), and lower pH at 10 h, 24 h, 2 days, and 3 days

(**Table 3**). Those suggested that in whole-plant corn silages with LAB suspension, the fermentation process and *Lactobacillus* succession were initiated early, and *Weissella*, *Lactococcus*, and *Leuconostoc* might grow rapidly and reduce pH (<4.5) for stimulating *Lactobacillus* to multiply and dominate bacterial community during the intense fermentation phase. Additionally, compared with CK and ESL, the CSL had higher *Lactobacillus* at 24 h and 3 days (**Figure 5** and **Supplementary Figure 2**) and lower pH at 24 h, 2 days, and 3 days (**Table 3**). Those indicated that the *Lactobacillus* in the LAB suspension prepared from whole-plant corn silage had greater adaptive capacity and reduced pH more efficiently in whole-plant corn silages than that in the LAB suspension prepared from *E. sibiricus* silage during this phase. Ali et al. (2020) reported that *Pediococcus* from red clover microbiota exhibited better adaption and dominated the bacterial community in red clover silage at 3 days of ensiling; Ohshima et al. (1997) and Wang et al. (2009) showed that LAB in pre-fermented fluid prepared from ensiling material is more effective at improving the fermentation quality than that prepared from other forage sources. The results mentioned above indicated that the homologous LAB might be more familiar with the physicochemical properties of the silage than other sources of LAB and have more efficiency in promoting microbial succession and fermentation of silage during the early stage. In the study, during the intense fermentation phase, the abundances of *Klebsiella*, *Rahnella*, *Enterobacter*, *Serratia*, *Pantoea*, and *Erwinia* decreased or went up and then down in CK, CSL, and ESL (**Figure 5**); moreover, the coliform count began to reduce after 10 h with pH <5.2 (**Table 3**). Those genera belonged to *Enterobacteriaceae* or *Erwiniaceae* and might be inhibited in anaerobic and acidic conditions (pH <5.4) (McGarvey et al., 2013). Keshri et al. (2018) reported that in whole-plant corn silages without inoculant, *Klebsiella* decreased and *Serratia* increased from 3 h to 3 days after ensiling; *Acinetobacter* was the most dominant genus at 3 h. However, in the study, the *Pantoea* was the most dominant genus at 3 h (**Figure 5**).

The acidic and anaerobic conditions of silage are unsuitable for most of the microorganisms (Ávila and Carvalho, 2019) and might lead to a decrease in the counts of LAB, yeast, and bacteria in whole-plant corn silage with pH <4.0 during the stable phase (**Table 4**). Moreover, Guan et al. (2020) also found the reducing counts of LAB and yeast from 3 to 60 days in whole-plant corn silage. The LAB population dominated the bacterial community during the stable phase and *Lactobacillus* showed the highest abundance among bacterial genera (**Figure 6** and **Supplementary Figure 3**), which is similar to the results of previous studies in whole-plant corn silages (Keshri et al., 2018; Guan et al., 2020; Xu et al., 2020; Zeng et al., 2020). The abundance of *Lactobacillus* increased from 3 to 10 days in CK and CSL, and to 30 days in ESL, and then reduced in the present study (**Figure 6**). Similar dynamics were detected in whole-plant corn silages by Zeng et al. (2020). However, Keshri et al. (2018) and Xu et al. (2020) reported that the abundance of *Lactobacillus* increased during fermentation and was more than 90% in final silages; additionally, Guan et al. (2020) found that, from 3 days after ensiling, the abundance increased in the silage at 30°C, while it decreased at 45°C. In the study, during this phase, the other LAB genera (*Weissella*, *Leuconostoc*, *Lactococcus*, and *Pediococcus*) present as minor taxa with less than 5% of abundances, except for *Weissella* in ESL_3 days and ESL_60 days and *Leuconostoc* and *Lactococcus* in ESL_3 days (**Figure 6**). However, Keshri et al. (2018) detected *Weissella* and *Pediococcus* with considerable abundance from 2 to 30 days, and Guan et al. (2020) reported *Pediococcus* as one dominant bacterial genus from 3 to 14 days at 30°C. The different dynamics of bacterial community among those studies mentioned above might be due to the difference in the corn species, geographical location, mowing period, epiphytic microflora, storing temperature, and inoculants (McGarvey et al., 2013; Guan et al., 2020).

In the first 5 h after ensiling, *Enterobacteriaceae* and *Erwiniaceae* dominated bacterial community in whole-plant corm silage with LAB population as minor taxa pH >5.5 (**Supplementary Figure 4** and **Tables 2**, **3**). The abundance of *Klebsiella* and *Enterobacter* increased (**Figures 3**, **5**) because *Enterobacteriaceae* thrived in anaerobic and weak acidic condition (pH >5.4) (McGarvey et al., 2013). Additionally, *Enterobacteriaceae* could ferment water-soluble carbohydrates and lactic acid to acetic acid or other products in silage (Ni et al., 2017). Those might explain the correlation of *Klebsiella* and *Enterobacter* with pH and acetic acid (**Figure 7A**). The *Weissella*, *Lactococcus*, *Leuconostoc*, and *Lactobacillus* had negative correlation with pH (**Figures 7A,B**) and their abundances went up during this time (**Supplementary Table 1**). Nevertheless, the LAB population is minor taxa in the first 3 h and the abundance of *Lactobacillus* in LAB population decreased in the first 5 h (**Supplementary Figure 3B**). Those indicated that the activity of *Weissella*, *Lactococcus*, and *Leuconostoc* might be the main reason for reducing the pH in whole-plant corn silage in the first 5 h. From 5 to 24 h, the LAB population had a rapidly increasing abundance and dominated the bacterial community after 10 h (**Figure 5** and **Supplementary Figure 3**). *Weissella*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* had negative correlation with pH (**Figure 7C**). Additionally, *Weissella* had greater abundance than other LAB genera (**Figure 5** and **Supplementary Figure 3**). Those suggested that the reducing pH of whole-plant corn silage was due to the activity of *Weissella* cooperating with *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus* during this time. *Klebsiella*, *Pantoea*, *Enterobacter*, and *Sphingomonas* had a reducing abundance and a positive correlation with pH from 5 to 24 h (**Figures 5**, **7C**), indicating that their activity might be inhibited under the acidic condition (pH <5.4). After 24 h, the pH had negative correlation with *Lactobacillus* dominating the bacterial community and correlated positively with *Weissella*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Pantoea* (**Figures 5**, **6**, **7D** and **Supplementary Figure 3**). Those suggested that the activity of *Lactobacillus* caused the reducing pH, and the other LAB genera and *Pantoea* might be restrained effectively by the acidic environment (pH <4.2). Moreover, Xu et al. (2020) also detected a negative correlation between pH and *Lactobacillus* sp. in whole-plant corn silage. Based on the above analysis between the pH and LAB population, the LAB fermentation relay occurred from *Weissella*, *Lactococcus*, and *Leuconostoc* (LAB genera as minor taxa) to *Weissella*, *Lactococcus*, *Leuconostoc*,

Lactobacillus, and *Pediococcus* (LAB genera rising rapidly), and then to *Lactobacillus* (LAB genera dominating).

CONCLUSION

In conclusion, *Lactobacillus*, *Weissella*, and *Lachnoclostridium*_5 were the predominant bacterial genera in LAB suspension-CS; *Lactobacillus* dominated bacterial community in LAB suspension-ES. During the initial aerobic phase, the pH and the abundances of *Pantoea*, *Klebsiella*, *Rahnella*, *Erwinia*, and *Serratia* increased. During the intense fermentation phase, the pH decreased rapidly and the microbial counts increased exponentially; the most predominant bacterial genus shifted from *Pantoea* to *Weissella* and then to *Lactobacillus*; the LAB suspensions promoted microbial succession and LAB suspension-CS was more effective. During the stable phase, the pH and microbial counts decreased, and *Lactobacillus* dominated bacterial community. The LAB fermentation relay occurred from *Weissella*, *Lactococcus*, and *Leuconostoc* to *Weissella*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus*, and then to *Lactobacillus* during the anaerobic fermentation process of whole-plant corn silage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LS, CB, and YX designed the study and wrote the manuscript. LS, CB, HX, NN, YJ, GY, and SL performed the experiments. HX and YJ reviewed and edited the manuscript. LS, CB, and YX analyzed the data. YX funded and supervised the experiments. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.655095/full#supplementary-material

Supplementary Figure 1 | Relative abundances of aerobic **(A)** and facultatively anaerobic **(B)** bacteria in whole-plant corn silages. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage.

Supplementary Figure 2 | Difference in bacterial communities (genus level) among treatments at 3 h, 5 h, 10 h, 24 h, 2 day, and 3 day. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage.

Supplementary Figure 3 | Relative abundances of main lactic acid bacterial genera in bacterial community **(A)** and in lactic acid bacterial population **(B)** of whole-plant corn silages. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage.

Supplementary Figure 4 | Relative abundance of bacterial communities (family level) in whole-plant corn silages. CK, whole-plant corn silage without any lactic acid bacterial suspensions; CSL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from whole-plant corn silage; ESL, whole-plant corn silage treated with lactic acid bacterial suspensions prepared from *Elymus sibiricus* silage.

Supplementary Table 1 | Relative abundances (%) of main lactic acid bacterial genera in whole-plant corn silages in the first 5 h.

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Effects of Microbial Inoculation and Storage Length on Fermentation Profile and Nutrient Composition of Whole-Plant Sorghum Silage of Different Varieties

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This study aimed to assess the effects of a heterofermentative microbial inoculant and storage length on fermentation profile, aerobic stability, and nutrient composition in whole-plant sorghum silage (WPSS) from different varieties. Experiment 1, a completely randomized design with a 2 × 3 factorial treatment arrangement, evaluated microbial inoculation [CON (50 mL distilled water) or LBLD (*Lactobacillus plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g of fresh forage)] and storage length (14, 28, or 56 d) in forage WPSS. The LBLD silage had lower pH compared to CON, and greater concentrations of succinic acid, ethanol, 1,2-propanediol (1,2-PD), 1-propanol, 2,3-butanediol and total acids. After 56 d, lactic acid concentration was greater for CON, while acetic acid and aerobic stability were greater in LBLD silage. Experiment 2, a completely randomized design with a 2 × 3 factorial treatment arrangement, evaluated effects of microbial inoculation (same as experiment 1) and storage length (14, 28, or 56 d) in WPSS of three varieties [forage sorghum (Mojo Seed, OPAL, Hereford, TX), sorghum-sudangrass (Dyna-gro Seed, Fullgraze II, Loveland, CO, United States), or sweet sorghum (MAFES Foundation Seed Stocks, Dale, MS State, MS)]. The LBLD forage sorghum had greater acetic acid and 1,2-PD concentrations at 56 d and 28 d, respectively, but lower concentrations of propionic acid at 56 d and butyric acid at 14 and 28 d. Additionally, WSC concentration was greater for CON than LBLD at 28 d. Furthermore, CON sweet sorghum had greater lactic acid, propionic acid, and butyric acid concentrations. However, greater acetic acid and 1,2-PD were observed for LBLD sweet sorghum. The CON sweet sorghum had greater concentration of WSC and yeast counts. The CON sorghum sudangrass had greater lactic and butyric acid concentrations than LBLD at 14 d, but lower acetic

acid and 1,2-PD concentrations at 56 d. Yeast counts were greater for CON than LBLD sorghum sudangrass silage. Overall, results indicate inoculation of WPSS with *Lactobacillus plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074 improves heterofermentative co-fermentation allowing the accumulation of acetic acid concentration and increasing antifungal capacities and aerobic stability of WPSS.

Keywords: *L. buchneri*, *L. diolivorans*, variety, aerobic stability, 1,2-propanediol.

INTRODUCTION

Sorghum production is gaining popularity with dairy producers in the United States, especially in regions that experience drought, delayed planting, and high summer temperatures which limit corn production (Dann et al., 2008; Hasan et al., 2017). Recently, studies have been focused on improving sorghum characteristics as a forage crop, including increasing yield and nutritive value (Reddy and Reddy, 2003; Kertikov, 2007). With a wide range of types and varieties available, it is of ultimate importance to select adapted materials that require low inputs, could recover from drought, have high yield potential under dryland regions, and high forage quality.

Commonly used sorghum types, such as sorghum-sudangrass and forage sorghum, are popular with growers because of their flexible planting time, rapid growth, high yields, suitability in rotation systems, and high nutritive value (McDonald et al., 1991; Cothren et al., 2000). Another line of breeding focused on developing sweet sorghum materials for bioenergy use because of its adaptability, high dry matter (DM) yield, growth characteristics (Knoll et al., 2018), and high concentration of fermentable sugars (Zhang et al., 2016). Although data embracing its use as silage is lacking, the chemical composition suggests it may be suitable for silage production, as water-soluble carbohydrates (WSC) are the primary substrate utilized by lactic acid bacteria (LAB) for growth at the beginning phase of ensiling, leading to a drastic reduction in pH (Yang et al., 2006), which is essential for silage preservation.

Microbial inoculants are a frequently used tool by dairy producers trying to influence silage fermentation and improve aerobic stability (Krooneman et al., 2002; Kleinschmit and Kung, 2006; Kung et al., 2018), but literature is controversial. Typically, traditional homofermentative microbial inoculants (mainly producing lactic acid) have been the preferred choice of producers in the United States to increase the rate of sorghum silage fermentation. Nevertheless, the second generation of heterofermentative microbial inoculants producing lactic and antifungal acids (i.e., acetic and propionic acids) from the degradation of other fermentation byproducts offer greater advantages. These contain a combination of strains from different species such as *Lactobacillus diolivorans* and *L. buchneri* (Kung et al., 2018), which can increase aerobic stability in sorghum silage. Inoculants containing a combination of both strains could enhance acetic and propionic acid production, which are antifungal compounds that suppress yeast and mold growth. The bacteria *L. buchneri* produces 1,2-propanediol (1,2-PD) during the degradation of lactic acid into acetic acid (Oude Elferink et al., 2001), and *L. diolivorans* can utilize 1,2-PD as a carbon source for growth, producing propionic acid and 1-propanol as the main fermentation end products (Zielińska et al., 2017). Capitalizing on a co-fermentation with both microbial strains can result in the production of more antifungal acids, improving aerobic stability and silage fermentation. Although the use of *L. buchneri* is well-established, its combination with new strains is receiving renewed attention (Fernandes et al., 2020; Ferrero et al., 2021).

Storage length is positively related to aerobic stability in silages inoculated with heterofermentative inoculants. Greater concentrations of acetic acid inhibit yeasts, resulting in enhanced aerobic stability (Kung et al., 2018), but the conversion of lactic acid into acetic acid was thought to begin after approximately 30 to 60 d of storage length (Driehuis et al., 1999; Muck et al., 2018). More recent studies demonstrated the onset of acetic acid production may begin as early as 15 d of storage (Fernandes et al., 2020), depending on bacterial strain and silo conditions. Besides, producers are often challenged with silage shortages, requiring silos to be opened before ideal circumstances would normally permit. In 2019, for example, abnormally wet spring conditions delayed corn planting for many producers, and for some prevented it altogether, leading to a record 11.4 million acres of corn unplanted in the U.S. (Schnepf, 2020). Conditions like these, and others such as limited growing area, create difficulties for producers without enough silage carryover from previous years. Delayed planting can encourage producers to turn to sorghum crops because of their more rapid maturation, which allows them to be harvested before corn.

Therefore, two experiments were conducted to assess the effects of adding heterofermentative microbial inoculants in whole-plant sorghum silage (WPSS). The objective of experiment 1 was to examine the effect of a microbial inoculant containing *L. plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074 and storage length on the fermentation profile, aerobic stability, and nutrient composition of WPSS. The objective of experiment 2 was to determine the effects of microbial inoculation with *L. plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074, and storage length on fermentation profile, nutrient composition and NDF ruminal disappearance of WPSS made from forage sorghum, sweet sorghum, and sorghum sudangrass. We hypothesized that microbial inoculation would improve the fermentation profile and aerobic stability of WPSS, and that this response would

increase with storage length and be of greater magnitude in varieties with greater soluble sugar concentrations.

MATERIALS AND METHODS

Experiment 1

This experiment was carried out in North Florida during the summer growing season of 2019. A completely randomized design with a 2 × 3 factorial arrangement of treatments was used to evaluate the effect of microbial inoculation [CON (50 mL distilled water) or LBLD (Provita Supplements Inc., Mendota Heights, MN, *Lactobacillus plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g of wet forage)] and storage length (14, 28, or 56 d) in WPSS.

Crop Establishment, Harvesting, and Ensiling

Sorghum forage (Ascend BMR MS Sorghum; DFA Farm Supplies, Syracuse, NY) was obtained from a commercial dairy farm (Alliance Dairies, Trenton, FL, United States) grown during the summer season. Fertilizer, pesticide, and herbicide applications were applied according to protocols established by Alliance Dairies and harvest timing was based on the farm harvest schedule. Forage was harvested from four locations (used as replication; 4 mini-silos per treatment combination [microbial inoculant by storage length]) within the same field on October 29th, 2019 at approximately 22% DM with a self-propelled forage harvester (Claas of America, Omaha, NE, united States) using a theoretical length of cut of 17 mm, without a kernel processor and without internal inoculant application. To ensure no contamination from previous microbial inoculant residue in the harvester, approximately 3 m of forage was harvested and then discarded.

Microbial inoculation rates were based on counts determined by pour plating on Man, Rogosa, Sharpe agar (Oxoid, Blasingstoke, United Kingdom). Microbial inoculant was diluted in distilled water targeting 300,000 CFU/g wet forage of *Lactobacillus plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074 (Provita Supplements Inc., Mendota Heights, MN, United States), applied and hand-mixed with chopped forage immediately before ensiling. Those not treated with LBLD were treated with distilled water in the same proportion (CON). Forage was packed in laboratory scale silos (20 L plastic buckets) considered as experimental units at a density of approximately 180 kg of DM/m^3. After packing, buckets were closed and sealed with tape to avoid aerobic exposure until reaching the targeted fermentation lengths (14, 28, or 56 d). Therefore, the experiment consisted of 6 treatments (two microbial inoculations × three storage lengths) and 24 silos (four replications per treatment combination).

Chemical Analysis

Fresh, uninoculated forage samples were collected for each of the four locations to establish a baseline for nutritive value, DM, pH and yeast, and mold counts. An undried and unground sample of 20 g was diluted 10-fold (mass basis) in 0.1% peptone water (Oxoid CM0090), blended for 60 s in a high-speed stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, OH, United States), and filtered through two layers of cheese cloth to extract forage juice. Forage extract was collected, and pH was immediately measured in duplicate using a pH meter (Corning model 12, Corning Scientific Instruments, Medfield, MA, United States). In addition, a separate sample of forage extract was added to a 50 mL plastic tube to evaluate yeast and mold enumeration via a pour plating method in a 10-fold serial dilution on malt agar (Difco 211220) acidified with 85% lactic acid. Agar plates were incubated at 32℃ for 48 h for yeast, and an additional 72 h for mold counts. Yeast and mold counts were evaluated on the same plates and separated by visually examining the growth of colonies. Fresh samples from each plot were dried in a forced-air oven set at 60℃ for 48 h to determine DM content, and sufficiently dried to be ground to pass through a 1-mm sieve using a Wiley Mill (Thomas Scientific, Swedesboro, NJ) and sent to Rock River Laboratory Inc. (Watertown, WI) for chemical characterization. Absolute DM was determined by oven-drying at 105°C for 3 h (method 2.2.2.5; National Forage Testing Association, 1993). Samples were analyzed for DM, crude protein (CP; method 990.03; AOAC International, 2012), ether extract (EE; method 920.39; AOAC International, 2012), starch (Hall, 2015), water-soluble carbohydrates (WSC; Dubois et al., 1956), and ash (method 942.05, AOAC International, 2012). Neutral detergent fiber was determined using α-amylase and sodium sulfite (aNDF; method 2002.04, AOAC International, 2012).

After experimental silos reached their assigned storage length, buckets were opened, and the top 10 cm of material was discarded. Immediately after opening, the material was homogenized, and a sub-sample of approximately 250 g was collected and frozen at −20°C for subsequent fermentation profile determination. In addition, samples were collected to evaluate DM content, dried and ground as described previously. Undried samples were collected and used to evaluate yeast and mold counts as described previously. After sample collection was completed, remaining silage (approximately 6 kg) for each individual silo was mixed and placed back in the experimental silo with two temperature sensors (HOBO temperature data logger 64 k; Onset Computer Corp., Cape Cod, MA, United States) placed in the geometric center of the bucket to evaluate aerobic stability. Sensors recorded the temperature every 30 min, and three additional sensors were used to monitor room temperature in the event of temperature fluctuations. Room temperature averaged 22.4℃ ± 1.14 among all periods of aerobic stability measurements. Buckets were covered with two layers of cheesecloth to prevent drying and silos were left exposed to aerobic conditions for 240 h. Aerobic stability was defined as the number of hours until silo temperature increased 2℃ above the baseline silo temperature.

Both, dried, and frozen samples were sent to Rock River Laboratory Inc. (Watertown, WI, United States) for analysis of nutrient composition and fermentation profile. Samples ensiled for 14, 28 and 56 d were analyzed for pH, organic acids, and fermentation byproducts (1,2-PD, 1-propanol, 2,3-butanediol, 2-butanol and ethanol). Twenty grams of undried, unground sample was diluted 10-fold (mass basis) in double distilled water, blended for 30 s in a high-speed blender, and filtered

through a filter funnel with a 2-mm pore size screen to extract silage juice. The extract was collected, and pH was immediately measured using a pH meter (Thermo-Orion Dual Star; Thermo Fisher Scientific Inc.) fitted with a glass pH electrode (Thermo-Orion 9172BNWP; Thermo Fisher Scientific Inc.). After pH was measured, the extract was centrifuged (750 × g) for 20 min at 25°C, and the supernatant was combined with 1.0 mL of calcium hydroxide solution and 0.5 mL of copper sulfate solution and re-centrifuged as described previously. The supernatant was analyzed for organic acids, and alcohols using high-performance liquid chromatography with an isocratic pump, auto sampler, column heater, refractive index detector (1515, 2707, heater, and 2414 respectively; Waters Corporation, Milford, MA, United States), and a reverse-phase ion exclusion column (Bio-Rad Aminex HPX-876H; Bio-Rad Laboratories, Hercules, CA). Flow rate and temperature were set at 0.6 mL/min for 40 min and 35°C, respectively. The mobile phase used was 0.015 N H_2SO_4/0.25 mM EDTA.

Statistical Analysis

Data were analyzed as a completely randomized design with a 2 × 3 factorial arrangement of treatments, with laboratory silos as experimental units, using PROC GLIMMIX of SAS (version 9.4; SAS Institute Inc., Cary, NC, United States) according to the statistical equation:

$$Y_{ij} = \mu + I_i + S_j + IS_{ij} + e_{ij},$$

with $e_{ij} \approx N(0, \sigma_e^2)$, where Y_{ij} is the value of the dependent variable, μ is the overall mean, I_i is the fixed effect of microbial inoculant (i = 1 and 2), S_j is the fixed effect of storage length (j = 1 to 3), IS_{ij} is the fixed interaction effect, e_{ij} is the residual error, N stand Gaussian distribution, $\sigma^2{}_e$ is the residual variance. Degrees of freedom were corrected by Kenward and Roger (1997) method. If significant, interaction effects were partitioned using the SLICE option to study the effect of microbial inoculation within each day of storage length. Significance was declared at $P < 0.05$.

Experiment 2

This experiment was carried out at the University of Florida Plant Science Research and Education Unit (Citra, FL, United States). A completely randomized design with a 2 × 3 factorial arrangement of treatments was used to evaluate the effect of microbial inoculation [CON (50 mL distilled water) or LBLD (Provita Supplements Inc., Mendota Heights, MN; *Lactobacillus plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g of wet forage)] and storage length (14, 28, or 56 d) in WPSS made from forage sorghum, sweet sorghum, and sorghum sudangrass.

Crop Establishment, Harvesting, and Ensiling

Forage sorghum, sorghum-sudangrass, and sweet sorghum were planted in July 2019, at 75,000, 122,000, and 75,000 plants.ha^{-1} seeding rates following companies' specifications, respectively, in an experimental irrigated field of 4 plots (considered as replication). Varieties were seeded in 4 rows of 6 m length each, spaced at 76 cm centers by a John Deere MaxEmerge Plus 170 4-row planter (Moline, IL, United States). Fertilizer applications consisted of 303 kg.ha^{-1} nitrogen, 237 kg.ha^{-1} potassium, 63 kg.ha^{-1} phosphorus, 40 kg.ha^{-1} sulfur, 18 kg.ha^{-1} magnesium, 11 kg.ha^{-1} manganese, 4.5 kg.ha^{-1} zinc divided in starter in furrow, 2 applications side-dressed and a final application applied through overhead irrigation. Pesticide application included 1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine (Atrazine, Syngenta, Lone Tree, IA, United States), Pendimethaline Penoxaline (Prowl, Basf, Research Triangle Park, NC, United States), and Metolachlor (Dual, Syngenta, Lone Tree, IA), at planting for weed control; Tebuconazole (TebuStar, Albaugh LLC, Ankeny, IA, United States) and Pyraclostrobin (Headline, Basf, Research Triangle Park, NC, United States) at 76 cm plant height, and Pyraclostrobin and Metconazole (Headline Amp, Basf, Research Triangle Park, NC, United States) at tasseling for fungal disease control. Insecticide application comprised of Chlorantraniliprole (Coragen, FMC Philadelphia, PA, United States), Cyhalothrin (Besiege, Syngenta, Lone Tree, IA), Lambda-cyhalothrin (Warrior, Syngenta, Lone Tree, IA, United States), and Flubendiamide (Belt, Bayer CropScience, Research Triangle Park, NC, United States) divided into 6 applications, following the guidelines established by the University of Florida/IFAS.

After tasseling, plants were monitored weekly for DM content and harvested at a targeted DM of 32%. Varieties were harvested individually on different days as plants tested at the threshold of 32% DM concentration. All plots from a variety were harvested on the same day. At harvest, in November 2019, plants from the 2 middle rows of each plot were hand-cut to a 25-cm stubble, in a 3-m continuous section and immediately processed with a single-row silage chopper (Model #707 SN: 245797; CNH Industrial America LLC, Burr Ridge, IL, United States) using a theoretical length of cut of 17 mm and without a kernel processor.

Microbial inoculation rates were based on LAB counts determined in experiment 1. Microbial inoculant was mixed and combined with sorghum forage as described before. Approximately 900 g of material was placed in nylon-polyethylene standard barrier vacuum pouches silos (0.09 mm thickness, 25.4 × 35.6 cm; Doug Care Equipment Inc., Springville, CA, United States), which were the experimental units, vacuum-sealed using an external clamp vacuum machine (Bestvac; distributed by Doug Care Equipment), and were randomly assigned to be stored for 14, 28, or 56 d. All silos were filled and sealed within 2 h after harvest, weighed then stored at room temperature in the dark until reaching the assigned storage length. Therefore, the experiment consisted of 6 treatments (two microbial inoculations × three storage lengths) per variety and 72 total mini-silos (four replications per treatment).

Chemical Analysis

Fresh samples from each plot of each variety were analyzed for DM, pH and yeast and mold counts as described previously. Additionally, dried samples were ground as described previously, and sent to Rock River Laboratory Inc. (Watertown, WI, United States) for chemical characterization (DM, CP, EE, starch, WSC, NDF, and ash). At opening, silos were weighed, and unground sample of 20 g was diluted 10-fold (mass basis) in 0.1% peptone water (Oxoid CM0090), blended for 60 s in a high-speed

stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, OH), and filtered through two layers of cheese cloth to extract forage juice. The extract was collected, and pH was measured as described previously. Forage extract was centrifuged (750 × g) for 20 min at 25°C, and the supernatant (40 mL) was combined with 0.4 mL of 50% sulfuric acid solution and re-centrifuged as previously described. The supernatant was kept in a freezer (−20°C) for subsequent analysis of organic acids and 1,2-PD. The concentrations of organic acids and 1,2-PD were determined using high-performance liquid chromatography (Merck Hitachi Elite La-Chrome, Hitachi L2400, Tokyo, Japan) as described by Muck and Dickerson (1988). For the organic acids analysis, a Bio-Rad Aminex HPX-87H ion exclusion column (300 × 7.8 mm id; Bio-Rad Laboratories, Hercules, CA) was used in an isocratic elution system containing 0.015 M sulfuric acid in the mobile phase of the high-performance liquid chromatography attached to an ultraviolet light detector (wavelength 210 nm; L-2400, Hitachi) using a flow rate of 0.7 mL/min during 40 min at 45°C. For 1,2-PD analysis, the same system and column were used running 0.005 M sulfuric acid in the mobile phase of the high-performance liquid chromatography attached to a Refractive Index detector (L-2490, Hitachi) using a flow rate of 0.6 mL/min during 40 min at 45°C. Unacidified forage juice extract was also evaluated for yeast and mold counts as described in experiment 1. The recovery of DM was calculated through the methodology proposed by Jobim et al. (2007), according to the equation:

$$\text{DM recovery} = \frac{(\text{FO} \times \text{DMO})}{(\text{FS} \times \text{DMS})}$$

where FO is the forage weight at opening, DMO is the dry matter concentration of the forage at opening, FS is the initial forage weight during ensiling, DMS is the initial dry matter concentration of the forage during ensiling.

A silage sample of 50 g was dried in a forced-air oven set at 60℃ for 48 h and ground as described previously. Fermented samples were sent to Rock River Laboratory Inc. (Watertown, WI) for chemical analysis. Samples were analyzed for absolute DM, CP, EE, starch, WSC, ash and NDF as described previously, as well as borate-phosphate-soluble CP (SP; Krishnamoorthy et al., 1982).

Additional dried silage samples were ground to pass through a 6-mm sieve using a Wiley Mill for ruminal in situ NDF disappearance. Ruminal *in situ* procedures were conducted at the University of Florida (Gainesville, FL, United States) under a protocol approved by the University of Florida, Institute of Food and Agricultural Sciences, and Animal Care Research Committee (protocol #201709849). A sample of 5 g was placed at Dacron polyester cloth bags (R1020, 10 × 20 cm and 50 ± 10 microporosity; Ankom Technology, Macedon, NY, United States) and incubated for 30 h in the rumen of three ruminally cannulated, mid-lactation, multiparous Holstein cows. Cows were fed a diet containing (DM basis) corn silage (38.2%), alfalfa hay (4.0%), dry ground shelled corn (27.3%), soybean meal (14.5%), citrus pulp (9.1%), minerals and supplements (6.8%). Additionally, two empty bags were incubated for correction of bag infiltration or losses. After the incubation period, bags were removed from the rumen, soaked in cold water for approximately 15 min to stop microbial activity, rinsed in a washing machine using the rinse mode and spin cycle, set with room temperature water for a 30-min cycle (Roper RTW4516F*, Whirlpool Corp., Benton Harbor, MI, United States), dried in a forced-air oven at 60° C for 72 h, and initial and residual NDF contents were analyzed with the percentage of difference considered as NDF disappearance.

Statistical Analysis

Data were analyzed separately for each variety as a completely randomized design with a 2 × 3 factorial arrangement of treatments with laboratory silos as the experimental units and using the PROC GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc, Cary, NC, United States) according to the statistical equation:

$$Y_{ijk} = \mu + I_j + S_k + IS_{jk} + e_{ijk},$$

with $e_{ijk} \approx N\ (0, \sigma_e^2)$, where Y_{ijk} is the value of the dependent variable, μ is the overall mean,, I_j is the fixed effect of microbial inoculant (j = 1 and 2), S_k is the fixed effect of storage length (k = 1 to 3), IS_{jk}, is the fixed interaction effect, e_{ijk} is the residual error, N stand Gaussian distribution, σ_e^2 is the residual variance. Degrees of freedom were corrected by Kenward and Roger (1997) method. If significant, interaction effects were partitioned using the SLICE option to study the effect of microbial inoculation within each day of storage length. Significance was declared at $P < 0.05$.

RESULTS

Main effects are discussed only if there were no significant interaction effects detected.

Experiment 1

Nutrient composition, pH, and yeast and mold counts of fresh sorghum forage are presented in **Table 1**. Effects of microbial inoculation and storage length on fermentation profile of whole-plant sorghum silage are in **Table 2**. Concentrations of butyric acid and 2-butanol were not detected among any treatments. The pH of silage was affected by microbial inoculant and was lower (P = 0.001) in LBLD treated silages (3.86) than CON (3.95). Total acid concentration was affected by microbial inoculant and storage length ($P \leq 0.01$). Total acid concentration was 11.5% DM for LBLD silage, while CON was 10.6% DM. Additionally, total acid concentration was greater after 56 d (12.5 % DM) compared to other storage lengths (10.3% DM, on average). An interaction between microbial inoculant × storage length was observed (P = 0.05) for the concentration of lactic acid, which was similar between microbial inoculants at 14 and 28 d, but lower for LBLD than CON after 56 d of storage. Similarly, acetic acid concentration was affected by the interaction of microbial inoculant × storage length (P = 0.01), with similar concentrations in LBLD and CON silages after 14 d of storage (1.5% of DM, on average), but greater for LBLD compared to CON silage after 28 (2.1 vs. 1.3% of DM, respectively) and 56 d (4.0 vs. 1.7% of DM, respectively). Succinic acid was affected by microbial inoculant and was greater (P = 0.02) for

TABLE 1 | Nutrient composition, pH, and yeast and mold counts of fresh, uninoculated whole-plant sorghum forage in experiments 1 and 2.

Item[1]	Experiment 1	Experiment 2		
	Forage sorghum	Sorghum-sudangrass	Sweet sorghum	Forage sorghum
DM, % of as fed	22.1 ± 0.60	34.9 ± 1.70	30.8 ± 1.75	30.6 ± 1.49
pH	5.84 ± 0.10	5.65 ± 0.19	5.95 ± 0.25	6.30 ± 0.08
WSC, % DM	11.2 ± 2.08	12.1 ± 1.13	34.1 ± 0.87	14.9 ± 2.17
CP, % DM	6.6 ± 0.33	6.5 ± 0.59	5.0 ± 1.84	7.9 ± 0.23
EE, % DM	2.9 ± 0.19	2.6 ± 0.80	1.6 ± 0.70	2.6 ± 0.38
NDF, % DM	59.4 ± 1.49	64.7 ± 1.21	49.2 ± 0.32	49.7 ± 1.17
Starch, % DM	4.10 ± 0.87	5.10 ± 0.79	15.3 ± 4.27	21.2 ± 0.79
Ash, % DM	5.90 ± 0.96	4.04 ± 0.20	2.97 ± 0.40	3.49 ± 0.21
Yeast, log CFU/g	4.18 ± 0.79	8.62 ± 0.21	9.36 ± 0.35	10.90 ± 0.42
Mold, log CFU/g	4.30 ± 0.51	2.20 ± 4.40	6.00 ± 4.00	2.90 ± 3.80

[1]*DM, dry matter; WSC, water-soluble carbohydrates; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber.*

TABLE 2 | Effect of microbial inoculation and storage length on the fermentation profile of whole-plant sorghum silage in experiment 1.

Item[1]	pH	Total acids, % DM	Lactic acid, % DM	Acetic acid, % DM	Propionic acid, % DM	Succinic acid, % DM	Ethanol, % DM	1,2 propanediol, % DM	1 propanol, % DM	2,3 butanediol, % DM
d 14										
CON	3.96	10.2	8.8	1.3	0.1	0.09	0.7	0.0	0.0	0.0
LBLD	3.88	10.6	8.5	1.8	0.2	0.11	1.3	0.1	0.0	0.1
d 28										
CON	3.94	9.6	8.2	1.3[b]	0.1	0.08	0.9	0.0	0.0	0.0
LBLD	3.86	10.8	8.4	2.1[a]	0.2	0.12	1.4	0.7	0.2	0.2
d 56										
CON	3.94	11.9	10.0[a]	1.7[b]	0.1	0.09	0.6	0.0	0.0[b]	0.0
LBLD	3.84	13.0	8.5[b]	4.0[a]	0.4	0.14	1.4	0.8	0.6[a]	0.4
Storage length means										
d 14	3.92	10.4[z]	8.6	1.5	0.1	0.11	1.0	0.1	0.0	0.1
d 28	3.90	10.2[z]	8.3	1.7	0.1	0.10	1.1	0.3	0.1	0.1
d 56	3.89	12.5[y]	9.2	2.9	0.3	0.11	1.0	0.4	0.3	0.2
Microbial inoculation means										
CON	3.95	10.6	9.0	1.4	0.1	0.08	0.7	0.0	0.0	0.0
LBLD	3.86	11.5	8.4	2.7	0.2	0.12	1.4	0.5	0.3	0.2
SEM[2]	0.02	0.41	0.35	0.35	0.17	0.03	0.31	0.33	0.11	0.09
P-value										
SL	0.26	0.001	0.02	0.001	0.51	0.71	0.75	0.31	0.01	0.06
MI	0.001	0.01	0.06	0.001	0.16	0.02	0.01	0.01	0.001	0.001
SL × MI	0.83	0.47	0.05	0.001	0.73	0.75	0.63	0.31	0.01	0.06

[a,b]*Means with different superscripts within the same day denote an effect of microbial inoculation within that day.*
[y,z]*Means with different superscripts denote differences among storage lengths.*
[1]*MI, effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL, effect of storage length (14, 28, and 56 d).*
[2]*Greatest standard error of the mean.*

LBLD (0.12% DM) than CON silage (0.08% DM). No fixed or interaction effects ($P \geq 0.16$) were observed for propionic acid concentration.

Both the concentration of ethanol ($P = 0.01$; 1.4% and 0.7% DM, respectively) and 1,2-PD ($P = 0.01$; 0.5% and 0.0% DM, respectively) were greater for LBLD silages compared to CON silages. Additionally, an interaction between microbial inoculant × storage length was observed for 1-propanol. There was no 1-propanol detected at 14 d for any treatment, while LBLD treated silage had ($P = 0.01$) 0.6% DM after 56 d of storage. The concentration of 2,3-butanediol was affected by microbial inoculant and

was greater (P = 0.001) for LBLD (0.2 % DM) than CON silages (0.0% DM).

Effects of microbial inoculation and storage length on nutrient composition, yeast count, and aerobic stability of whole-plant sorghum silage are in **Table 3**. Starch concentration and mold counts were also evaluated. For starch, because a male sterilized sorghum variety was used for this experiment, all treatment averages were less than 1% DM (0.68% DM, on average) and no fixed or interaction effects were observed ($P > 0.05$). Additionally, mold counts were lower than 2 log CFU/g. Therefore, these variables are not included in tables or figures. An interaction between microbial inoculant and storage length was observed ($P = 0.01$) for aerobic stability. Aerobic stability was similar between microbial inoculant treatments at 14 d, but 47 h and 156 h longer for LBLD treated silage than CON after 28 and 56 d of storage, respectively.

Overall, microbial inoculant and storage length had little effect on the nutrient composition of whole-plant sorghum silage, with no effects ($P > 0.07$) observed for WSC, EE, CP, and NDF concentrations in addition to yeast counts. An interaction between microbial inoculant and storage length was observed ($P = 0.01$) for DM. Concentration of DM was similar at 14 d, but slightly greater (1.0%-unit, on average) for CON than LBLD at 28 and 56 d of storage. A microbial inoculant effect ($P = 0.01$) was observed for ash concentration which was greater for CON (7.1% DM) than LBLD silage (6.1% DM).

Experiment 2

Nutrient composition, pH, and yeast and mold counts of fresh, uninoculated sorghum varieties are in **Table 1**. Furthermore, similar to experiment 1, because all mold counts were less than 2 log CFU/g, this variable is not included in any tables.

Effects of microbial inoculation and storage length on fermentation profile of whole-plant forage sorghum silage are in **Table 4**. No fixed or interaction effects ($P \geq 0.20$) were observed for silage pH. Lactic, acetic, and butyric acids in addition to 1,2-PD were affected by an interaction of microbial inoculant × storage length ($P \leq 0.05$). Lactic acid concentration was greater (11.3% DM) for CON silage than LBLD (4.7% DM) at 14 d of storage, but similar (10% DM, on average) at 28 and 56 d. Similarly, acetic acid concentration for CON silage was greater (2.6% vs. 1.0% DM, respectively) at 14 d, similar at 28 d, but lower (1.7% vs. 5.7% DM, respectively) than LBLD after 56 d of storage. Likewise, butyric acid only had detectable concentrations for CON silages after 14 d (0.5% DM) and 28 d (0.3% DM) of storage. The concentration of 1,2-PD was greater for LBLD silage than CON after 28 (0.9 and 0.0% DM, respectively) and 56 d (3.1 and 0.0% DM, respectively) of storage, but not 14 d. For propionic

TABLE 3 | Effect of microbial inoculation and storage length on the nutrient composition, yeast counts, and aerobic stability of whole-plant sorghum silage in experiment 1.

Item[1]	DM, % as fed	Ash, % DM	WSC, % DM	EE, % DM	CP, % DM	NDF, % DM	Yeast, log CFU/g	Aerobic stability, h
d 14								
CON	21.3	6.9	2.5	2.9	7.3	48.5	2.65	136
LBLD	21.2	5.6	3.0	3.0	6.9	61.8	5.40	157
d 28								
CON	21.1[a]	7.6	1.9	2.7	7.3	62.2	3.88	76[b]
LBLD	20.4[b]	6.2	2.6	2.9	7.6	61.5	2.47	123[a]
d 56								
CON	21.2[a]	6.9	1.9	3.2	7.1	61.1	5.08	35[b]
LBLD	19.6[b]	6.6	2.5	3.1	7.5	64.4	3.14	191[a]
Storage length means								
d 14	21.3	6.2	2.7	3.0	7.1	55.1	4.03	147
d 28	20.8	6.9	2.2	2.8	7.4	61.8	3.18	100
d 56	20.4	6.8	2.2	3.1	7.3	62.7	4.11	113
Microbial inoculation means								
CON	21.2	7.1	2.1	2.9	7.2	57.3	3.88	82
LBLD	20.4	6.1	2.7	3.0	7.3	62.5	3.67	156
SEM[1]	0.32	0.46	0.57	0.21	0.23	5.82	1.46	24.59
P-Value								
SL	0.01	0.12	0.49	0.11	0.26	0.37	0.62	0.04
MI	0.001	0.01	0.16	0.43	0.46	0.28	0.82	0.001
SL × MI	0.01	0.25	0.99	0.61	0.07	0.47	0.07	0.01

[a,b] *Means with different superscripts within the same day denote an effect of microbial inoculation within that day.*
[1] *MI: effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).*
[2] *Greatest standard error of the mean.*

TABLE 4 | Effect of microbial inoculation and storage length on the fermentation profile of whole-plant forage sorghum silage in experiment 2.

Item[1]	pH	Lactic acid, % DM	Acetic acid, % DM	Propionic acid, % DM	Butyric acid, % DM	1,2 propanediol, % DM
d 14						
CON	3.54	11.3[a]	2.6[a]	0.0	0.5[a]	0.0
LBLD	3.51	4.7[b]	1.0[b]	0.0	0.0[b]	0.3
d 28						
CON	3.50	11.2	2.5	0.0	0.3[a]	0.0[b]
LBLD	3.50	12.3	3.5	0.0	0.0[b]	0.9[a]
d 56						
CON	3.60	9.6	1.7[b]	0.7	0.0	0.0[b]
LBLD	3.65	8.7	5.7[a]	0.0	0.0	3.1[a]
Storage length means						
d 14	3.53	8.0	1.8	0.0	0.2	0.2
d 28	3.50	11.8	3.0	0.0	0.2	0.4
d 56	3.62	9.1	3.7	0.3	0.0	1.6
Microbial inoculation means						
CON	3.55	10.7	2.3	0.2	0.3	0.0
LBLD	3.55	8.6	3.4	0.0	0.0	1.4
SEM[2]	0.07	0.86	0.38	–	0.10	0.23
P-value						
SL	0.20	0.001	0.001	–	0.08	0.0001
MI	0.92	0.01	0.01	–	0.08	0.0001
SL × MI	0.85	0.001	0.001	–	0.05	0.0001

[a,b] *Means with different superscripts within the same day denote an effect of microbial inoculation within that day.*
[1] *MI, effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).*
[2] *Greatest standard error of the mean.*

acid, no treatments had detectable concentration except for CON silage after 56 d (0.7% DM).

Effects of microbial inoculation and storage length on the nutrient composition of whole-plant forage sorghum are presented in **Table 5**. Concentration of DM was greater ($P = 0.04$) at 14 d of storage length (29.2% as fed) than 28 and 56 d (27.6% as fed, on average). Similarly, ash concentration was greater ($P = 0.01$) at 28 d (4.1% DM) than other storage length treatments (3.6% DM, on average). An interaction between microbial inoculant × storage length ($P = 0.05$) was observed for WSC concentration; CON silage (3.6% DM) was greater than LBLD (1.7% DM) at 28 d, but similar at 14 and 56 d. The concentration of EE was greater ($P = 0.001$) for LBLD (2.4% DM) than CON silage (1.6% DM). Soluble CP concentration was affected by both microbial inoculation ($P = 0.001$) and storage length ($P = 0.02$). Soluble CP was greater in CON silage (53.2% DM) compared with LBLD (46.1% DM). Additionally, the concentration of soluble CP was lower after 14 d of storage (46.3% DM) compared with other storage lengths (51.3% DM, on average). Starch concentration was affected by storage length and was lower ($P = 0.01$) after 28 d of storage (19.2% DM) compared with other storage lengths (23.7% DM, on average). No fixed or interaction effects ($P \geq 0.08$) were observed for DM recovery, concentrations of CP and NDF, NDF disappearance and yeast counts.

Effects of microbial inoculation and storage length on fermentation profile of whole-plant sweet sorghum silage are in **Table 6**. An interaction between microbial inoculant × storage length ($P = 0.001$) was observed for pH, which was greater for CON (3.69) than LBLD (3.24) at 14 d of storage, but similar between microbial inoculants at 28 and 56 d. Lactic acid concentration was affected by microbial inoculant and storage length ($P \leq 0.04$). Lactic acid concentration was 7.9% DM for LBLD silage, while CON was 10.1% DM. Moreover, lactic acid concentration was lower after 14 d (5.6% DM) in comparison to other storage lengths (10.7% DM, on average). An interaction between microbial inoculant × storage length ($P \leq 0.03$) was observed for acetic, propionic, and butyric acids along with 1,2-PD. Concentration of acetic acid did not differ between microbial inoculants at 14 and 28 d but was greater for LBLD (5.0% DM) compared with CON (3.7% DM) at 56 d. Propionic acid concentration did not differ at 14 d, but was greater for CON than LBLD treatment at 28 (0.5 vs. 0.0% of DM, respectively) and 56 d (0.6 vs. 0.0% of DM, respectively). The butyric acid concentration for CON (0.7% DM) was greater than LBLD (0.0% DM) silage at 14 d, but not at 28 and 56 d. No differences were detected for 1,2-PD concentration at 14 d, while LBLD was greater than CON

TABLE 5 | Effect of microbial inoculation and storage length on the nutrient composition and yeast counts of whole-plant forage sorghum silage in experiment 2.

Item[1]	DM, % as fed	DM recovery, % DM	Ash, % DM	WSC, % DM	EE, % DM	CP, % DM	Sol.CP, % CP	NDF, % DM	Starch, % DM	NDF disappearance, % NDF	Yeast, log CFU/g
d 14											
CON	29.7	97.4	3.3	3.8	1.4	8.7	48.6	46.8	22.8	26.4	0.60
LBLD	28.7	94.8	3.7	3.7	2.1	8.3	44.0	46.3	24.0	25.4	0.85
d 28											
CON	27.6	90.6	4.1	3.6[a]	1.5	8.4	55.4	49.3	18.5	22.0	0.64
LBLD	27.5	91.5	4.1	1.7[b]	2.7	8.5	46.4	50.8	19.9	28.2	1.03
d 56											
CON	27.3	90.1	3.9	2.1	2.0	8.4	55.5	44.9	22.4	22.1	0.00
LBLD	27.9	92.2	3.5	2.7	2.5	8.9	47.8	48.3	25.8	24.3	0.00
Storage length means											
d 14	29.2[y]	96.1	3.5[z]	3.8	1.7	8.5	46.3[z]	46.6	23.4[y]	25.9	0.72
d 28	27.5[z]	91.0	4.1[y]	2.7	2.1	8.5	50.9[y]	50.0	19.2[z]	25.1	0.84
d 56	27.6[z]	91.2	3.7[z]	2.4	2.2	8.7	51.7[y]	46.6	24.1[y]	23.2	0.00
Microbial inoculation means											
CON	28.2	92.7	3.8	3.2	1.6	8.5	53.2	47.0	21.2	23.5	0.41
LBLD	28.0	92.8	3.8	2.7	2.4	8.6	46.1	48.5	23.3	26.0	0.62
SEM[1]	0.68	2.58	0.14	0.49	0.22	0.22	1.79	1.66	1.42	2.26	0.60
P-value											
SL	0.04	0.11	0.01	0.03	0.12	0.56	0.02	0.08	0.01	0.48	0.34
MI	0.79	0.95	0.93	0.26	0.001	0.63	0.001	0.30	0.10	0.19	0.67
SL × MI	0.51	0.63	0.07	0.05	0.37	0.15	0.47	0.52	0.71	0.30	0.95

[a,b] *Means with different superscripts within the same day denote an effect of microbial inoculation within that day.*
[y,z] *Means with different superscripts denote differences among storage lengths.*
[1] *MI: effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).*
[2] *Greatest standard error of the mean.*

silage after 28 (0.5 vs. 0.0 % of DM, respectively) and 56 d (3.1 vs. 0.0% of DM, respectively) of storage.

Effects of microbial inoculation and storage length on nutrient composition and yeast count of whole-plant sweet sorghum silage are in **Table 7**. Overall, microbial inoculant and storage length had minimal effects on the nutrient composition of whole-plant sweet sorghum silage, with no fixed or interaction effects ($P \geq 0.09$) observed for DM, DM recovery, concentrations of ash and soluble CP, and NDF disappearance. An interaction between microbial inoculant × storage length ($P \leq 0.02$) was observed for WSC and EE concentrations. Water-soluble carbohydrates concentrations were similar between microbial inoculants at 14 and 28 d, but greater for CON (21.4% DM) than LBLD silage (15.6% DM) at 56 d. The EE concentration of LBLD was greater than CON at 14 (1.7 vs. 0.9% DM, respectively) and 28 d (1.8 vs. 1.0% DM, respectively), but did not differ at 56 d. A microbial inoculant effect ($P = 0.02$) was observed for CP concentration which was greater for LBLD (4.4% DM) than CON silage (4.0% DM). A storage length effect ($P = 0.03$) was observed for NDF concentration, which was lower after 14 d (45.9% DM) compared with other storage lengths (49.1% DM, on average). Similarly, starch concentration was affected by storage length ($P = 0.02$) and was greater at 14 d (11.7% DM) than 28 and 56 d (9.3% DM, on average). Furthermore, yeast counts were affected by microbial inoculation ($P = 0.001$) and were greater for CON (2.8 log CFU/g) than LBLD silage (0.0 log CFU/g).

Effects of microbial inoculation and storage length on the fermentation profile of whole-plant sorghum sudangrass silage are presented in **Table 8**. Propionic acid concentration was evaluated, but not detected among any treatments. No fixed or interaction effects ($P \geq 0.09$) were observed for silage pH. An interaction of microbial inoculant × storage length was detected ($P \leq 0.05$) for lactic, acetic, and butyric acids as well as 1,2-PD concentration. Lactic acid concentration was greater for CON (9.3% DM) than LBLD (4.8% DM) silage at 14 d, but similar at 28 and 56 d. Acetic acid concentration was greater for CON silage in comparison to LBLD at 14 d (2.5 vs. 1.1% of DM, respectively), similar at 28 d, but lower at 56 d (2.0 vs. 3.3% of DM, respectively). The concentration of butyric acid was greater for CON (0.6% DM) than LBLD (0.0% DM) silage at 14 d but did not differ at 28 and 56 d. The 1,2-PD concentration was greater for LBLD silage than CON after 28 (0.3 vs. 0.0 % of DM, respectively) and 56 d (0.6 vs. 0.0 % of DM, respectively).

Effects of microbial inoculation and storage length on the nutrient composition of whole-plant sorghum sudangrass silage are presented in **Table 9**. The concentration of EE was lower

TABLE 6 | Effect of microbial inoculation and storage length on the fermentation profile of whole-plant sweet sorghum silage in experiment 2.

Item[1]	pH	Lactic Acid, % DM	Acetic Acid, % DM	Propionic Acid, % DM	Butyric Acid, % DM	1,2 Propanediol, % DM
d 14						
CON	3.69[a]	7.2	2.3	0.0	0.7[a]	0.0
LBLD	3.24[b]	4.1	1.2	0.2	0.0[b]	0.0
d 28						
CON	3.35	11.2	3.9	0.5[a]	0.0	0.0[b]
LBLD	3.29	9.5	3.7	0.0[b]	0.0	0.5[a]
d 56						
CON	3.30	11.9	3.7[b]	0.6[a]	0.0	0.0[b]
LBLD	3.40	10.3	5.0[a]	0.0[b]	0.0	3.1[a]
Storage length means						
d 14	3.46	5.6[z]	1.8	0.1	0.3	0.0
d 28	3.32	10.3[y]	3.8	0.2	0.0	0.3
d 56	3.35	11.1[y]	4.4	0.3	0.0	1.5
Microbial inoculation means						
CON	3.45	10.1	3.3	0.4	0.2	0.0
LBLD	3.31	7.9	3.3	0.1	0.0	1.2
SEM[2]	0.04	1.20	0.41	0.10	0.01	0.15
P-value						
SL	0.01	0.001	0.001	0.06	0.001	0.001
MI	0.001	0.04	0.98	0.001	0.001	0.001
SL × MI	0.001	0.77	0.03	0.001	0.001	0.001

[a,b] *Means with different superscripts within the same day denote an effect of microbial inoculation within that day.*
[y,z] *Means with different superscripts denote differences among storage lengths.*
[1] *MI: effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).*
[2] *Greatest standard error of the mean.*

(P = 0.01) in CON (2.4% DM) compared with LBLD (2.8% DM) silage. An interaction between microbial inoculant × storage length (P = 0.03) was only observed for NDF concentration, which was similar between treatments at 14 and 28 d, but greater in LBLD silage after 56 d (65.4% DM) compared with CON (59.7% DM). Yeast counts were affected by both microbial inoculant (P = 0.05) and storage length (P = 0.02). Yeast counts were greater in CON silage (2.0 CFU/g) than LBLD silage (0.7 CFU/g). Additionally, yeast counts decreased as storage length increased, from 2.2 CFU/g after 14 d to 0.0 CFU/g after 56 d of storage. No fixed or interaction effects ($P \geq 0.06$) were observed for DM recovery, concentrations of DM, ash, WSC, CP, soluble CP, and starch, or NDF disappearance.

DISCUSSION

Both LBLD and CON silages in each experiment had sufficient concentrations of lactic acid for proper forage preservation (Kung et al., 2018). These same authors reported that lower DM silages have the potential to produce greater concentrations of lactic acid, explaining the high concentrations observed in experiment 1. Interestingly, the concentration of lactic acid production observed in CON silages suggests that the epiphytic microbial population had an adequate amount of lactic acid bacteria to promote a beneficial fermentation in all CON treatments for both experiments.

In the current experiments, greater acetic acid concentration in experiment 1 combined with the main effect of inoculation in experiment 1 and interaction effects in experiment 2 on 1,2-PD for LBLD silage compared to CON suggests that *L. buchneri* DSM 12856 was actively involved in a heterofermentative activity after a short period of storage. However, overall, it appears there was a less pronounced heterofermentative activity of *L. diolivorans* DSM 32074, as evidenced by the lack of effect of inoculation on propionic acid in experiment 1. Conversely, because of the heterofermentative activity of the inoculant, inoculating with *L. plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074 increased total acid production in sorghum silage in experiment 1. Thus, LBLD silages showed a lower pH compared to CON. However, in experiment 2, pH was only affected by an interaction of microbial inoculant and storage length in sweet sorghum. Nonetheless, sweet sorghum pH only differed with microbial inoculant after 14 d of storage, further supporting that in general, CON treatments had sufficient epiphytic LAB to promote a desirable fermentation.

TABLE 7 | Effect of microbial inoculation and storage length on the nutrient composition and yeast counts of whole-plant sweet sorghum silage in experiment 2.

Item[1]	DM, % as fed	DM recovery, % DM	Ash, % DM	WSC, % DM	EE, % DM	CP, % DM	Sol.CP, % CP	NDF, % DM	Starch, % DM	NDF disappearance, % NDF	Yeast, log CFU/g
d 14											
CON	26.9	93.5	3.0	22.5	0.9[b]	4.0	65.5	46.5	11.7	22.2	2.1
LBLD	26.9	93.4	3.2	22.3	1.7[a]	4.1	58.2	45.3	11.6	23.1	0.0
d 28											
CON	27.0	92.2	3.2	19.2	1.0[b]	4.0	59.6	48.3	9.5	23.0	3.7
LBLD	26.9	93.3	3.2	18.0	1.8[a]	4.3	62.1	50.1	9.1	23.9	0.0
d 56											
CON	27.2	93.8	3.2	21.4[a]	2.1	4.0	66.5	46.3	10.8	22.9	2.4
LBLD	26.0	89.7	3.1	15.6[b]	1.7	4.9	63.9	51.5	7.7	21.1	0.0
Storage length means											
d 14	26.9	93.5	3.1	22.4	1.3	4.0	61.9	45.9[z]	11.7[y]	22.7	1.1
d 28	26.9	92.8	3.3	18.6	1.4	4.2	60.9	49.2[y]	9.3[z]	23.4	1.9
d 56	26.6	91.8	3.2	18.5	1.9	4.5	65.2	48.9[y]	9.2[z]	22.0	1.2
Microbial inoculation means											
CON	27.1	93.2	3.1	21.0	1.3	4.0	64.0	47.0	10.6	22.7	2.8
LBLD	26.6	92.2	3.3	18.6	1.7	4.4	61.4	49.0	9.5	22.7	0.0
SEM[1]	0.65	1.16	0.22	0.97	0.19	0.21	2.15	1.27	0.86	2.37	0.85
P-Value											
SL	0.88	0.36	0.62	0.001	0.01	0.13	0.14	0.03	0.02	0.83	0.61
MI	0.42	0.29	0.46	0.01	0.01	0.02	0.17	0.08	0.11	0.99	0.001
SL × MI	0.59	0.09	0.85	0.02	0.01	0.16	0.10	0.07	0.19	0.81	0.61

[a,b] *Means with different superscripts within the same day denote an effect of microbial inoculation within that day.*
[y,z] *Means with different superscripts denote differences among storage lengths.*
[1] *MI: effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).*
[2] *Greatest standard error of the mean.*

Kleinschmit and Kung (2006) reported that the addition of *L. buchneri* might lead to a 0.1 to 0.2 pH units increase due to a conversion of lactic acid to acetic acid, 1,2-PD, and ethanol (Oude Elferink et al., 2001). Previous research (EFSA FEEDAP Panel, 2016) reported that inoculation with *L. diolivorans* alone reduced pH in silages from several different forages, corroborating our results from experiment 1. Increasing the production of total acids, such as in experiment 1, may decrease pH despite LBLD not having a greater lactic acid content, which is the strongest acid in the silo. Moreover, in experiment 1 storage length also increased total acid concentration, demonstrating that the extent of fermentation occurring in the silo increases with time.

In both experiments, LBLD silages had greater concentrations of 1,2-PD compared to CON silages. This is primarily due to the heterofermentative activity of *L. buchneri*, which can produce 1,2-PD by degrading lactic acid while also creating ethanol as a byproduct of this process (Oude Elferink et al., 2001). However, low propionic acid concentrations at later storage lengths in LBLD silages might be due to delayed involvement of *L. diolivorans* DSM 32074 in heterofermentative activity or the production of 1-propanol by *L. diolivorans* DSM 32074 instead of utilizing the pathway producing propionic acid (Zhang et al., 2010; Zielińska et al., 2017). For example, the rapid decline of pH in LBLD sweet sorghum in experiment 2 may be attributed to the activity of *L. plantarum* DSM 21762, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074in the presence of high WSC concentrations. Furthermore, it has also been reported that *L. diolivorans* can produce acetic acid, 1,2-PD and ethanol in petri dishes containing glucose and 1,2-PD (Schein et al., 2018). Hence, it may be hard to discern in these experiments whether *L. diolivorans* DSM 32074 was engaged in the production of acetic acid or more focused on homofermentative activity. Experiment 1 demonstrates *L. diolivorans* DSM 32074 was involved in heterofermentative activity because of the increase in 1-propanol, but the low 1-propanol concentrations suggest a lower overall activity using this pathway. Interestingly, in the presence of glucose, Schein et al. (2018) did not observe propionic acid production by *L. diolivorans*, suggesting silo conditions may encourage homofermentative activity or heterofermentative activity that yields acetic acid, 1,2-PD, and 1-propanol. These same authors observed in petri dishes studies with mannitol and 1,2-PD as potential substrates, there was propionic acid production. These findings suggest extreme low WSC concentrations may be needed for *L. diolivorans* to utilize the pathway that yields propionic acid. This supports our premise that under specific

TABLE 8 | Effect of microbial inoculation and storage length on the fermentation profile of whole-plant sorghum-sudangrass silage in experiment 2.

Item[1]	pH	Lactic acid, % DM	Acetic acid, % DM	Butyric acid, % DM	1,2 propanediol, % DM
d 14					
CON	3.83	9.3[a]	2.5[a]	0.6[a]	0.0
LBLD	3.69	4.8[b]	1.1[b]	0.0[b]	0.2
d 28					
CON	3.74	7.3	1.7	0.3	0.0[b]
LBLD	3.73	6.8	1.5	0.1	0.3[a]
d 56					
CON	3.78	7.6	2.0[b]	0.0	0.0[b]
LBLD	3.64	9.4	3.3[a]	0.0	0.6[a]
Storage length means					
d 14	3.76	7.1	1.8	0.3	0.1
d 28	3.74	7.0	1.6	0.2	0.2
d 56	3.71	8.5	2.6	0.0	0.3
Microbial inoculation means					
CON	3.79	8.1	2.0	0.3	0.0
LBLD	3.69	7.0	2.0	0.04	0.4
SEM[2]	0.07	1.19	0.45	0.09	0.08
P-Value					
SL	0.80	0.39	0.08	0.01	0.03
MI	0.09	0.28	0.93	0.01	0.001
SL × MI	0.60	0.05	0.02	0.01	0.03

[a,b]Means with different superscripts within the same day denote an effect of microbial inoculation within that day.
[1]MI: effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).
[2]Greatest standard error of the mean.

silo conditions, *L. diolivorans* DSM 32074 may favor the 1-propanol pathway or the production of acetic acid over the pathway that produces propionic acid as observed in experiment 1. Additionally, although there was an effect of inoculation and storage length, the greater propionic acid concentrations found in CON sweet sorghum in experiment 2 were likely from epiphytic microbes, such as *Clostridium propionicum* (Kung et al., 2018). Overall, further research is needed to evaluate the effect of *L. diolivorans* DSM 32074 in the silo to highlight the potential production of acetic acid or demonstrate pathway preferences between 1-propanol and propionic acid production. Conversely, differences in WSC concentrations highlight the potential for heterofermentative bacteria, such as those used in this trial, to begin heterofermentative activity at different times depending on what sorghum variety is used.

While the onset of heterofermentative activity by *L. diolivorans* DSM 32074 or *L. buchneri* DSM 12856 cannot be isolated in these experiments, a shift from lactic to acetic acid occurred after the traditional 30 to 60 d lag reported by Muck et al. (2018). Acetic acid production in experiment 1 was greater in LBLD silage and increased with storage length, confirming there was *L. buchneri* DSM 12856 activity beginning at approximately 28 d (Oude Elferink et al., 2001). In experiment 2, all varieties were similarly affected by microbial inoculant and storage. However, in forage sorghum and sudangrass, CON silages had greater levels of acetic acid after 14 d of storage. This increase in acetic acid concentration without a corresponding increase in 1,2-PD could be indicative of fermentation by undesirable bacteria, such as enterobacteria, *Clostridia* or heterolactic bacteria (McDonald et al., 1991). In forage sorghum and sudangrass, there may have been fermentation by these undesirable microbes early in the silo for CON treatments, while the high WSC in sweet sorghum likely encouraged the rapid onset of homofermentative activity by LAB early in the silo and discouraged fermentation by undesirable bacteria for CON silage. Despite this, all varieties had a greater acetic acid concentration in LBLD after 56 d, as expected, after inoculation with *L. buchneri* (Oude Elferink et al., 2001).

Butyric acid is often considered a marker of undesirable fermentation and results from the fermentation of some bacteria such as *Clostridia* species (Kung et al., 2018). Although there was no effect on butyric acid observed in experiment 1 or sudangrass in experiment 2, there was an interaction effect in forage and sweet sorghum in experiment 2. Both varieties only had small concentrations detected in early storage lengths of CON silage and all detectable amounts were less than 1% DM. Although this indicates there may have been some undesirable fermentation, the small concentration does not

TABLE 9 | Effect of microbial inoculation and storage length on the nutrient composition and yeast counts of whole-plant sorghum-sudangrass silage in experiment 2.

Item[1]	DM, % as fed	DM Recovery, % DM	Ash, % DM	WSC, % DM	EE, % DM	CP, % DM	Sol.CP, % CP	NDF, % DM	Starch, % DM	NDF disappearance, % NDF	Yeast, log CFU/g
d 14											
CON	34.6	98.4	4.7	2.8	2.3	7.1	49.3	62.4	4.7	20.2	3.0
LBLD	33.7	97.4	4.6	2.2	2.6	7.0	50.4	61.1	4.2	21.4	1.4
d 28											
CON	33.8	96.9	4.4	3.6	2.3	6.8	57.5	61.8	3.8	24.9	2.9
LBLD	34.2	98.2	4.7	2.0	2.6	7.0	51.6	60.5	4.5	18.5	0.6
d 56											
CON	35.0	99.2	4.4	3.1	2.4	7.2	53.4	59.7[b]	5.3	19.8	0.0
LBLD	34.2	96.6	5.0	2.5	3.2	7.2	54.0	65.4[a]	4.8	18.3	0.0
Storage length means											
d 14	34.2	97.9	4.6	2.5	2.5	7.0	49.9	61.8	4.5	20.8	2.2[y]
d 28	34.0	97.5	4.6	2.8	2.5	6.9	54.6	61.2	4.1	21.7	1.8[y]
d 56	34.6	97.9	4.7	2.8	2.8	7.2	53.7	62.5	5.1	19.0	0.0[z]
Microbial inoculation means											
CON	34.5	98.1	4.5	3.2	2.4	7.0	53.4	61.3	4.6	21.6	2.0
LBLD	34.0	97.4	4.8	2.2	2.8	7.1	52.0	62.3	4.5	19.4	0.7
SEM[2]	0.71	0.85	0.16	0.58	0.18	0.35	2.08	1.40	0.71	1.95	0.76
P-Value											
SL	0.72	0.89	0.64	0.82	0.14	0.68	0.08	0.63	0.44	0.40	0.02
MI	0.47	0.29	0.08	0.06	0.01	0.87	0.41	0.39	0.85	0.18	0.05
SL × MI	0.58	0.10	0.14	0.63	0.40	0.83	0.20	0.03	0.62	0.17	0.33

[a,b]Means with different superscripts within the same day denote an effect of microbial inoculation within that day.
[y,z]Means with different superscripts denote differences among storage lengths.
[1]MI: effect of microbial inoculant (50 ml of distilled water or 300,000 CFU/g wet forage of L. plantarum DSM 21762, L. buchneri DSM 12856, and L. diolivorans DSM 32074 [Provita Supplements Inc., Mendota Heights, MN]); SL: effect of storage length (14, 28, and 56 d).
[2]Greatest standard error of the mean.

suggest a clostridial fermentation and is likely the result of fermentation by small numbers of these bacteria.

In LBLD silage in experiment 1, greater concentrations of succinic acid were observed. This may be because of the fermentation of lactic and citric acids, which has been observed for *L. plantarum* (Lindgren et al., 1989) and is included in the tested inoculant. Additionally, the effect of inoculation and storage on 2,3-butanediol in experiment 1 is likely the result of its production by the LAB included in the inoculant used in this study, as different species in this genus have been shown to produce this fermentation byproduct (Alan et al., 2018).

Silages with greater aerobic stability are desirable because of its resistance to deterioration and reduced DM losses at feedout (Wilkinson and Davies, 2012). In experiment 1, inoculation with *L. plantarum* DSM 21762*, L. buchneri* DSM 12856 and *L. diolivorans* DSM 32074 increased aerobic stability with increasing storage length, likely because of the increase in acetic acid production and, to a lesser extent the numerically greater propionic acid production. Aerobic stability is increased by the production or supplementation of antifungal acids, such as acetic and propionic acids (Kung et al., 2018). The presence of antifungal acids suppresses the growth of yeasts, which assimilate lactic acid and promote an increase in pH after silage is exposed to air (Wilkinson and Davies, 2012). The increase in pH allows other undesirable microbes to reproliferate in the silage, contributing to the greater DM losses after aerobic exposure.

Sorghum-sudangrass may have supplied less WSC for microbial activity compared to other varieties, whereas forage and sweet sorghum appears to provide adequate concentrations (Reddy and Reddy, 2003) that could be used by LAB, determining the rate of decline in pH (Davies et al., 1998). However, the possibly slower decline in pH for sorghum-sudangrass and in the case of sweet sorghum, the high concentration of WSC, might have contributed to yeast growth in CON silage, even during the anaerobic period (Ruxton et al., 1975). These results suggest that WSC content of sweet sorghum and sudangrass in the current trial may be favorable to lactate-assimilating yeasts, but further research is warranted to elucidate this premise. This finding highlights that further investigation to determine an upper and lower limit of carbohydrates for upcoming varieties used for silage is needed to discourage yeast growth during ensiling. In experiment 2, LBLD silages reduced yeasts counts in sorghum-sudangrass and sweet sorghum, likely due to the observed effects of microbial inoculation on acetic acid concentration. In forage sorghum, the yeast counts appear to be lower overall compared to the other varieties, possibly because there were no problems

related to WSC content as described previously in sweet sorghum and sudangrass, resulting in the lack of effect of microbial inoculant on yeast counts. A reduction in yeast counts is desirable because yeasts and molds can cause silage spoilage when exposed to oxygen (Muck et al., 2018). However, there was no effect of inoculation on yeast and no suppression of mold growth in experiment 1. Other studies performed on low DM (22 to 24%) corn and sorghum silages have reported additives containing *L. buchneri* inhibited mold growth with similar concentrations of acetic acid production (Filya, 2003). Based on the results in the current study, the lack of effect on yeast counts is not biologically obvious.

In addition, in experiment 2 microbial inoculation and storage length influenced the fermentation of forage sorghum by increasing SP with increasing storage length and decreasing SP with inoculation. Increasing SP indicates the breakdown of the protein matrix surrounding starch granules (Hoffman et al., 2011) with greater ensiling time (Ferraretto et al., 2015; Fernandes et al., 2020) such as in CON sorghum forage silage. Approximately 60% of proteolysis occurs due to bacterial enzymes in grain silages. Although the proteolytic activity of homofermentative LAB in silage is not known, heterofermentative inoculants may create conditions that favor proteolytic bacteria (Junges et al., 2017). Unexpectedly, the combination of *L. plantarum* DSM 21762*, L. buchneri* DSM 12856 and *L. diolivorans* DSM 32074 did not enhance concentrations of SP in comparison to CON silages. Moreover, minimal changes in starch content with greater storage length agree with the body of literature (Ferraretto et al., 2015; Fernandes et al., 2020). Greater ash concentration was observed in forage sorghum but is unlikely to be biologically significant. The small changes in EE concentration observed in this experiment, combined with the overall low concentration, suggest these effects are unlikely to be of biological significance. The concentration of WSC in experiment 2 was affected by interactions for both forage and sweet sorghum. In forage sorghum, the lower concentration in LBLD silage after 28 d of storage may be related to an increase in the rate of fermentation in LBLD beginning sometime after 14 d of storage. For sweet sorghum, the WSC concentration was much lower for LBLD than CON after 56 d of storage, suggesting a more substantial fermentation with greater production of acids. In experiment 1 there was a greater ash concentration in CON silage, but LBLD silages were only 0.9%-units lower. Therefore, the difference in ash concentration is again unlikely to be biologically significant or affect diet formulation.

Consequently, inoculation with LBLD led to a greater NDF content in sudangrass after 56 d of storage, which is known to be less digestible (Cherney et al., 1986). Besides these differences in carbohydrates, previous reports have shown similar NDF content with greater storage length than used in the present study (Sanderson, 1993; Der Bedrosian et al., 2012; Ferraretto et al., 2015), suggesting that heterofermentative inoculation's effect on fiber portion probably decreases with greater storage length. However, the mechanism behind the increase in NDF concentration with storage length in sweet sorghum may be related to the reduction in WSC content with storage length, increasing the relative proportion of fiber in the silo.

In experiment 1, the decrease in DM content with inoculation and storage length may be partially caused by *L. diolivorans* DSM 32074. It has been reported that water is produced as a byproduct of the degradation of 1,2-PD to 1-propanol or propionic acid (Zhang et al., 2010). Therefore, the numerical increase of propionic acid with inoculation and increasing storage length, along with the increase in 1-propanol concentration, probably contributed to water production in LBLD silage from the current study.

Results of this study indicate inoculating sorghum silage with a combination of *Lactobacillus plantarum* DSM 21762*, L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074 improves heterofermentative co-fermentation that allows for the accumulation of acetic acid concentration, increasing antifungal capacities of sorghum silage and thereby, its aerobic stability. Kung et al. (2018) indicated propionic acid concentrations greater than 0.3% DM is usually found in clostridial fermentations, likely a result of *Clostridium propionicum*. In the current study, high concentrations of propionic acid associated with short-term ensiling may have being from *L. diolivorans* DSM 32074 activity, or Clostridia might have outcompeted *L. diolivorans* DSM 32074 for substrate. Further research is warranted to elucidate these findings. Future research should focus on sequencing technologies to elucidate the preferential pathway for *L. diolivorans* to accelerate growth capacity in silage throughout the use of intermediates produced by *L. buchneri*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Florida, Institute of Food and Agricultural Sciences, Animal Care Research Committee.

AUTHOR CONTRIBUTIONS

LF contributed to conception and design of the study, as well as funding acquisition. ED and MP conducted the experiments, conducted the statistical analysis, and drafted the initial version of this manuscript. LG, JG, CH, CM, and MW participated in the experiments and editing of the manuscript. All authors contributed to manuscript revisions, read, and approved the submitted manuscript version.

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Natural Fermentation Quality and Bacterial Community of 12 *Pennisetum sinese* Varieties in Southern China

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This study investigated the fermentation quality of 12 varieties of *Pennisetum sinese* grown in different regions of Southern China. Following the production of silage from the natural fermentation of *P. sinese*, the interplay between the chemical composition, fermentation characteristics, environmental factors, and microbiome was examined to understand the influence of these factors on the fermentation quality of silage. The silage quality produced by most of the *P. sinese* was low; the pH value of the silage was high (4.26–4.86), whilst the lactic acid content was low (10.7–24.1 g/kg DM), with V-scores between 57.9 and 78.3. The bacterial alpha diversities of the 12 *P. sinese* silages were distinct. There was a predominance of undesirable bacteria (*Pseudomonas*, *Massilia*, and *Raoultella)*, which likely caused the poor fermentation quality. The chemical composition and fermentation characteristics of the silage were closely correlated with the composition of the bacterial community. Furthermore, environmental factors (precipitation, temperature, humidity, location) were found to significantly influence the microbiome of the silage. The results confirmed that silage produced from the natural fermentation of 12 different *P. sinese* varieties had significant variation in their bacterial communities. The difference in environmental factors, due to the *P. sinese* being grown in various locations across south china, greatly affected the bacterial community found in the silage and thus the fermentation quality. The specific cultivar used for the silage and the environment in which the cultivar is grown must therefore be considered before the initiation of production of silage in order to ensure a higher quality product.

Keywords: *Pennisetum sinese*, varieties, silage, fermentation quality, bacterial community, environmental factor

INTRODUCTION

Pennisetum sinese is a member of the grass family. It is adaptable and has a high biomass production rate; this has led to its widespread use in tropical/subtropical regions across the world (Zhao et al., 2019). *P. sinese* is primarily used for the production of feedstock for biofuels and animal feed; this is due to its high lignocellulosic biomass and excellent palatability (Li et al., 2014, 2019a,b, 2020; Lu et al., 2014; Wang et al., 2016; Shah et al., 2018). The demand for *P. sinese* is large, but production

is affected by seasonal harvest. The summer/rainy seasons see vigorous *P. sinese* growth, with a contrasting lack of growth during winter and times of drought; this leads to a shortage in its availability (Li et al., 2014). In order to combat this and meet the year-round demand, *P. sinese* needs proper pretreatment and conservation.

Pennisetum sinese has a high moisture content, making the plant susceptible to mildew and rot; this causes biomass decomposition and inefficient biotransformation. Those using *P. sinese* in tropical regions face considerable challenges regarding preservation and ineffective biotransformation, due to the hot and humid environment. Ensiling is a method that can conserve green plants by utilizing anaerobic fermentation. Ensiling can guarantee the quality of the feed for long term storage and has also been shown to be a promising pretreatment method in biofuel production (Wilkinson et al., 2003). Previous studies have focused primarily on the effect of additives to *P. sinese* silages, but little is currently understood regarding the effect of additives on the microbiome of the resultant silage (Li et al., 2014, 2018a,b, 2019a, 2020; Li L. et al., 2017; Wu et al., 2020). Currently, only a limited number of studies have examined the effects of additives on the fermentation quality and microbial diversity of *P. sinese* silage (Dong et al., 2020; Shah et al., 2020). It is of note that these studies have focused on specific *P. sinese* cultivars.

As previously mentioned, uncontrollable environmental factors challenge the production of silage in tropical areas (Bernardes et al., 2018). The significance of errant weather conditions has previously been examined in silage produced from the fermentation of Napier and Italian Ryegrass (Guo et al., 2014, 2015). In addition, found that environmental factors influenced the bacterial diversity of corn silage and thus altered the fermentation quality (Guan et al., 2018). Moreover, it has been reported that temperature can influence the bacterial diversity and fermentation quality of silage produced from *Moringa oleifera* (Wang et al., 2019a).

Pennisetum sinese is grown across multiple regions of southern china that differ greatly in climate. Therefore, the influence of environmental factors on silage quality and microbial diversity should be examined and any effects should be investigated in the multiple *P. sinese* cultivars that are used across the regions.

This study aimed to examine the differing bacterial communities and the fermentation of silages produced from 12 different *P. sinese* varieties. The *P. sinese* studied were grown across multiple regions in southern China; this allowed the experimental parameters to include influence of environmental factors (precipitation, temperature, humidity, location) on the silage microbiome across a broad spectrum of varieties of *P. sinese*.

MATERIALS AND METHODS

Silage Preparation

Twelve *P. sinese* varieties were grown at six different bases across southern China; the location and environmental factors related to these bases are shown in **Supplementary Table 1**. The *P. sinese* cultivars used in this study were planted on the 5th of April 2019 and then harvested on June 26th 2019. The *P. sinese* material was immediately chopped into small pieces (∼2 cm). Briefly, 200 g of each grass sample was vacuum-packed into plastic bags. A total of 36 bags (twelve varieties in triplicate) were prepared and incubated at 25–30°C for 30 days. The organic acid content and bacterial community of the silage was then determined.

Chemical Composition and Fermentation Analysis

Specimens were dried at 65°C for 2 days and passed through a 1.0 mm sieve before the chemical assay. The contents of dry matter (DM), crude protein (CP), organic matter (OM), and ether extracts (EE) were examined according to previously published work (AOAC, 1990). Moreover, the contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were assessed using a previously established method (Van Soest et al., 1991). Heat-stable amylase and sodium sulfite were adopted in the determination of NDF. Water-soluble carbohydrate (WSC) was determined according to a previously described method (Murphy, 1958).

The fermentation quality of the silage was determined using distilled water extracts. 50 g of wet silage was blended with 200 mL distilled water, before 24 h of incubation (4°C) and filtration. The pH, lactic acid, acetic acid, propionic acid, butyric acid, and ammonia-N concentrations were measured as previously established (Li et al., 2019b). To evaluate the silage quality the V-score was calculated using the formula: ammonia-N/Total N and organic acid (Masaki, 2001).

Microbial Community Analysis

The above-mentioned extracts were used for molecular analysis of the silage microbiome. Briefly, 20 mL filtrate was centrifuged at 12,000 *g*/min for 5 min, and the sediment was collected from the bottom. Microbial DNA was isolated from silage specimens using the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States), according to the manufacturer's instructions. The concentration and purity of the extracted DNA was assessed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States) and the integrity of the DNA was assessed by electrophoresis on a 1% agarose gel. A thermocycler PCR system (GeneAmp 9700, ABI, United States) and primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3–V4 hypervariable regions of the bacterial 16S rRNA gene. After the PCR products were purified and quantified, next-generation sequencing was carried out using an Illumina MiSeq 2500 platform (Illumina, Inc., San Diego, CA, United States). Paired-end reads of 250 bp were generated.

Tag assembly was carried out using filtered reads according to the following principles: the overlap between paired-end reads should be more than 10-bp and have less than a 2% mismatch. The unique tags were obtained by removal of redundant tags using MOTHUR software (Schloss et al., 2009).

Abundancy was determined using the resultant unique tags and high-quality reads were grouped into operational taxonomic units (OTUs) defined as having 97% similarity. Diversity metrics were determined using the core-diversity plug-in within QIIME2[1] (Callahan et al., 2016). The microbial diversity within an individual sample was assessed using the following alpha diversity indices: the Chao 1 richness estimator, Shannon diversity index, and Faith's phylogenetics diversity index. Beta diversity was analyzed to assess the structural variation of microbiota across *P. sinese* specimens. Subsequently, principal component analysis (PCA) was undertaken (Vázquez-Baeza et al., 2013). Appropriate methods were employed to identify the abundances of different bacterial strains amongst samples and groups as described previously (Segata et al., 2011). Unless specified above, parameters used in the genetic analysis were set as default. The heat map function of the R software[2] and genus information for the *P. sinese* silage were used to generate a heat map. The data were analyzed using the free online BMKCloud Platform[3]. The sequencing data was submitted to the National Center for Biotechnology Information Sequence Read Archive database under the BioProject accession number PRJNA624770.

Statistics

The impact of variety was investigated using one-way analysis of variance (SAS 9.3 software, SAS Institute Inc., Cary, NC, United States). Significant differences were compared using Duncan's multiple range tests, with $P < 0.05$ being regarded as statistically significant.

RESULTS

The Chemical Composition of Fresh *P. sinese*

The Chemical compositions of different fresh *P. sinese* samples are shown in **Table 1**. In this study, the *P. sinese* DM content ranged from 113.6 to 225.2 g/kg; the MZC and MOTT samples had the highest and lowest DM contents, respectively ($P < 0.05$). The CP contents ranged from 47.4 to 73.0 g/kg, with the MZC and JING samples ($P < 0.05$), having the highest and lowest CP values, respectively. The OM contents of all *P. sinese* samples were similar, above 900 g/kg, with no significant difference. The NDF content of all the samples ranged from 522.0 to 652.8 g/kg; the highest and lowest levels were found in the PURP and XC samples, respectively ($P < 0.05$). The ADF content of the *P. sinese* samples ranged from 230.6 to 391.8 g/kg; the highest and lowest levels were found in the MOTT and XC samples, respectively ($P < 0.05$). The WSC content of the *P. sinese* samples ranged from 53.8 to 93.4 g/kg, with the highest levels in the MIN and DY samples, respectively ($P < 0.05$). Overall, with the exception of the OM content, the chemical composition of the *P. sinese* samples varied greatly.

The Fermentation Property of *P. sinese* Silage

The fermentation properties of silage produced by different *P. sinese* varieties are shown in **Table 2**. There were significant differences in the pH levels, as well as the lactic acid, acetic acid, propionic acid, butyric acid, and ammonia-N content depending on the variety off *P. sinese* used to produce the silage ($P < 0.05$). The pH of the silages ranged from 4.36 to 4.86, with the silage produced by MIN and MZC *P. sinese* varieties having the highest and lowest pH levels, respectively ($P < 0.05$). The lactic acid content of all *P. sinese* silages ranged from 10.7 to 24.1 g/kg, with XC having the highest lactic acid content and AX the lowest ($P < 0.05$). The acetic acid content of the different silages ranged from 3.0 to 16.6 g/kg, with the silages produced by the MOTT and AX *P. sinese* samples having the highest and lowest levels, respectively ($P < 0.05$). The propionic acid content of all *P. sinese* silage samples ranged from 1.1 to 17.3 g/kg; the highest propionic acid content was found in the XC ($P < 0.05$), whilst the lowest propionic acid content was found in the MZC sample ($P < 0.05$). Meanwhile, the butyric acid content of all *P. sinese* silage samples ranged from 1.2 to 4.6 g/kg. The silage from the MIN sample had the highest butyric acid content with the PURP sample silage having the lowest ($P < 0.05$). Finally, the ammonia-N content of the *P. sinese* silages ranged from 44.5 to 67.2 g/kg, the highest ammonia-N content was found in the JING silage sample ($P < 0.05$), whilst the lowest

TABLE 1 | Chemical composition of 12 *P. sinese* varieties (g/kg DM^{-1}).

Variety	DM	CP	OM	NDF	ADF	WSC
MIN	209.1^{ab}	62.1^{b}	949.3^{a}	647.5^{a}	377.0^{b}	93.4^{a}
TW	124.3^{f}	54.6^{cd}	936.9^{a}	537.2^{d}	342.3^{bc}	96.3^{a}
MOTT	113.6^{g}	58.2^{bc}	941.2^{a}	608.5^{b}	391.8^{a}	68.8^{c}
KG	186.8^{c}	55.9^{c}	919.8^{a}	605.1^{b}	358.3^{b}	80.6^{b}
DY	181.5^{c}	58.3^{bc}	930.6^{a}	584.2^{bc}	333.7^{c}	53.8^{e}
HONG	129.8^{f}	65.9^{b}	917.3^{a}	575.4^{c}	356.9^{b}	71.0^{c}
JING	183.2^{c}	47.4^{f}	902.4^{a}	569.2^{c}	347.5^{bc}	73.6^{c}
AX	128.7^{f}	58.4^{bc}	936.7^{a}	525.7^{e}	230.6^{d}	57.2^{d}
ZJ	201.7^{b}	53.6^{d}	913.6^{a}	599.7^{b}	357.0^{b}	62.4^{d}
MZC	225.2^{a}	73.0^{a}	930.6^{a}	588.6^{bc}	366.0^{b}	90.3^{a}
PURP	145.0^{e}	51.7^{e}	946.1^{a}	652.8^{a}	360.8^{b}	60.1^{d}
XC	163.4^{d}	56.6^{c}	919.1^{a}	522.0^{e}	366.9^{b}	63.4^{d}
SEM	10.76	1.94	4.13	12.26	11.65	4.20
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

MIN, Pennisetum purpureum Schum. cv. Gui Min Yin; TW, Pennisetum purpureum Schumab ev. Taiwan; MOTT, Pennisetum purpureum cv. Mott; KG, Pennisetum purpureum × P. glaucum cv. Reyan No. 4; DY, Pennisetum purpureum K.; HONG, Pennisetum purpureum Schumab cv.; JING, Pennisetum purpureum K. Jingmu No. 1; AX, Pennisetum purpureum K.; ZJ, Pennisetum americanum × Pennisetum purpureum; MZC, Pennisetum glaucum; PURP, Pennisetum purpureum cv. Purple; XC, Pennisetum purpureum Schumach. DM, dry matter, CP, crude protein, OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water-soluble carbohydrate; SEM, standard error of means. Means within the same column with different letters are significantly different (P < 0.05).

[1] https://docs.qiime2.org/2019.1/
[2] http://www.r-project.org/
[3] www.biocloud.net

in the ZJ sample ($P < 0.05$). The V-Scores of all the *P. sinese* silages ranged from 57.9 to 78.3, the highest V-Score was found in the AX sample silage with the lowest in the MIN sample ($P < 0.05$). All the naturally fermented *P. sinese* silages in this study had a low V-score, which suggests that the silage quality was unsatisfactory. In addition, the results showed large variability in the quality of fermentation and V-scores across the different *P. sinese* silage samples.

Bacterial Community of *P. sinese* Silage

A total of 2,677,149 raw reads were generated, with an average of 71,884 raw tags per *P. sinese* sample. After data processing, there was an average of 71,423 clean tags and 60,717 effective tags obtained from the silage samples.

Table 3 shows the alpha diversity of the silage bacterial communities. The OTU, Shannon, Simpson, Ace, and Chao 1 indices of microbial diversity were affected by the variety of samples (**Table 3**). The OTUs, Shannon, Simpson, Ace, and Chao 1 values ranged from 383 to 500, 1.39 to 3.54, 0.08 to 0.45, 367.57 to 476.25, 368.29 to 490.58, respectively. In conclusion, the variety of *P. sinese* greatly affected the microbiome of the resultant silage. **Figure 1A** describes the microbial community at the phylum level. *Proteobacteria, Firmicutes, Bacteroidetes*, and Actinobacteria were dominant in all *P. sinese* samples. To further investigate the effects of *P. sinese* variety on the resultant microbial community following ensiling, the bacterial structures of *P. sinese* silages at the genus level were examined (**Figure 1B**). The 10 genera with the highest relative abundance were: *Pseudomonas*, *Massilia*, *Raoultella*, *Pedobacter*, *Sphingobacterium*, *Acinetobacter*, *Megamonas*, *Lactobacillus*, *Enterococcus*, and *Stenotrophomonas*. The present study shows that *Pseudomonas* is one of the most abundant genera of bacteria present in the silages produced by differing *P. sinese* varieties. It is proposed that *Pseudomonas* contributed to the poor *P. sinese* silage quality.

The linear discriminant analysis (LDA) effect size (LEfSe) method was used to assess the differences in the microbiome content between the silages produced by the different *P. sinese* varieties, as well as to explore the specific bacterial community of each silage variety (LDA score >4.0). **Figure 2** shows that the variety of *P. sinese* used to produce the silage had a dramatic impact on the resultant microbial community. *Bacteroidales* was the most abundant order in the silage of the AX *P. sinese* variety, *Verticia* was the most abundant genus and *Lactobacillus casei* was the most abundant species in the DY silage. *Actinomyces* was the most abundant species in the HONG silage, *Sphingobacterium* was the most abundant genus in the KG silage whilst *Enterococcus*, *Paenibacillus*, and *Citrobacter* were the most abundant genera in the MZC silage. *Flavobacterium* was the most abundant genus and *Pedobacter Ellin108* the most abundant species in the MIN silage. *Corynebacteriales* and *Flavobacteriales* were the most abundant orders, *Sphingobacteriaceae*, *Nocardiopsaceae*, *Weeksellaceae*, and *Rhizobiaceae* the most abundant families and *Enterococcus*, *Pedobacter*, *Chryseobacterium*, and *Pseudochrobactrum* were the most abundant genera in the MOTT silage. Conversely, *Lactococcus*, and *Bradyrhizobium* were the most abundant genera in the PURP silage, whilst in the XC silage *Micrococcales* was the most abundant order, *Paeniglutamicibacter* the most abundant genus and *Pseudomonas lundensis* was the most abundant species. Finally, *Corynebacterium_1* was the most abundant genus in ZJ silage. It is proposed that these microorganisms could be used as

TABLE 2 | Fermentation characteristics of the silage from 12 varieties of *P. sinese* (g/kg DM^{-1}).

Variety	pH	Lactic acid	Acetic acid	Propionic acid	Butyric acid	Ammonia -N	V-Score
MIN	4.86^{a}	16.6^{c}	3.8^{g}	11.1^{cd}	3.6^{a}	66.7^{a}	57.9^{d}
TW	4.57^{b}	15.4c	7.6e	12.6c	2.4^{bc}	54.2^{bc}	70.0^{b}
MOTT	4.75^{ab}	21.6^{b}	16.6^{a}	15.7^{b}	2.3^{bc}	60.8^{b}	69.4^{b}
KG	4.39^{cd}	13.7^{d}	5.7^{f}	9.5^{d}	2.7^{b}	45.8^{d}	68.4^{b}
DY	4.68^{ab}	12.4^{e}	5.0^{f}	6.5^{e}	1.9^{d}	49.2^{c}	77.5^{a}
HONG	4.54^{bc}	12.2^{e}	9.4^{d}	1.4^{f}	3.9^{a}	53.5^{bc}	61.3^{cd}
JING	4.81^{a}	16.1^{c}	13.9^{b}	12.4^{c}	3.1^{ab}	67.2^{a}	61.8^{cd}
AX	4.62^{b}	10.7^{f}	3.0^{g}	2.0^{f}	2.2^{bc}	58.8^{b}	78.3^{a}
ZJ	4.46^{c}	12.4^{e}	8.1^{e}	6.0^{e}	2.6^{b}	44.5^{d}	69.9^{b}
MZC	4.36^{d}	20.4^{b}	10.6^{cd}	1.1^{f}	2.6^{b}	48.3^{c}	71.7^{b}
PURP	4.63^{b}	12.8^{e}	6.3^{f}	13.0^{c}	2.2^{bc}	51.0^{c}	72.2^{b}
XC	4.50^{bc}	24.1^{a}	11.5^{c}	17.3^{a}	3.4^{a}	49.8^{c}	62.8^{cd}
SEM	0.05	1.23	1.19	1.61	0.18	2.21	1.84
P-value	<0.05	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

MIN, Pennisetum purpureum Schum. cv. Gui Min Yin; TW, Pennisetum purpureum Schumab ev. Taiwan; MOTT, Pennisetum purpureum cv. Mott; KG, Pennisetum purpureum × P. glaucum cv. Reyan No. 4; DY, Pennisetum purpureum K.; HONG, Pennisetum purpureum Schumab cv.; JING, Pennisetum purpureum K. Jingmu No. 1; AX, Pennisetum purpureum K.; ZJ, Pennisetum americanum × Pennisetum Purpureum; MZC, Pennisetum glaucum; PURP, Pennisetum purpureum cv. Purple; XC, Pennisetum purpureum Schumach; SEM, standard error of means. Means within the same column with different letters are significantly different (P < 0.05).

TABLE 3 | The bacterial alpha diversity of silage from 12 *P. sinese* varieties.

Variety	OTUs	Shannon	Simpson	ACE	Chao 1	Coverage
MIN	489	2.97	0.25	461.46	468.44	0.99
TW	491	2.51	0.22	463.36	466.13	0.99
MOTT	486	3.54	0.08	469.19	477.53	0.99
KG	489	2.59	0.30	464.63	474.68	0.99
DY	419	2.59	0.16	460.27	382.71	0.99
HONG	500	1.96	0.45	469.16	473.36	0.99
JING	497	3.42	0.16	476.25	481.40	0.99
AX	495	3.23	0.20	475.82	490.58	0.99
ZJ	469	2.64	0.17	434.45	423.30	0.99
MZC	383	1.39	0.44	367.57	368.29	0.99
PURP	421	2.27	0.23	430.28	395.26	0.99
XC	406	2.53	0.17	368.59	372.94	0.99

MIN, Pennisetum purpureum Schum. cv. Gui Min Yin; TW, Pennisetum purpureum Schumab ev. Taiwan; MOTT, Pennisetum purpureum cv. Mott; KG, Pennisetum purpureum × P. glaucum cv. Reyan No. 4; DY, Pennisetum purpureum K.; HONG, Pennisetum purpureum Schumab cv.; JING, Pennisetum purpureum K. Jingmu No. 1; AX, Pennisetum purpureum K.; ZJ, Pennisetum americanum × Pennisetum Purpureum; MZC, Pennisetum glaucum; PURP, Pennisetum purpureum cv. Purple; XC, Pennisetum purpureum Schumach.

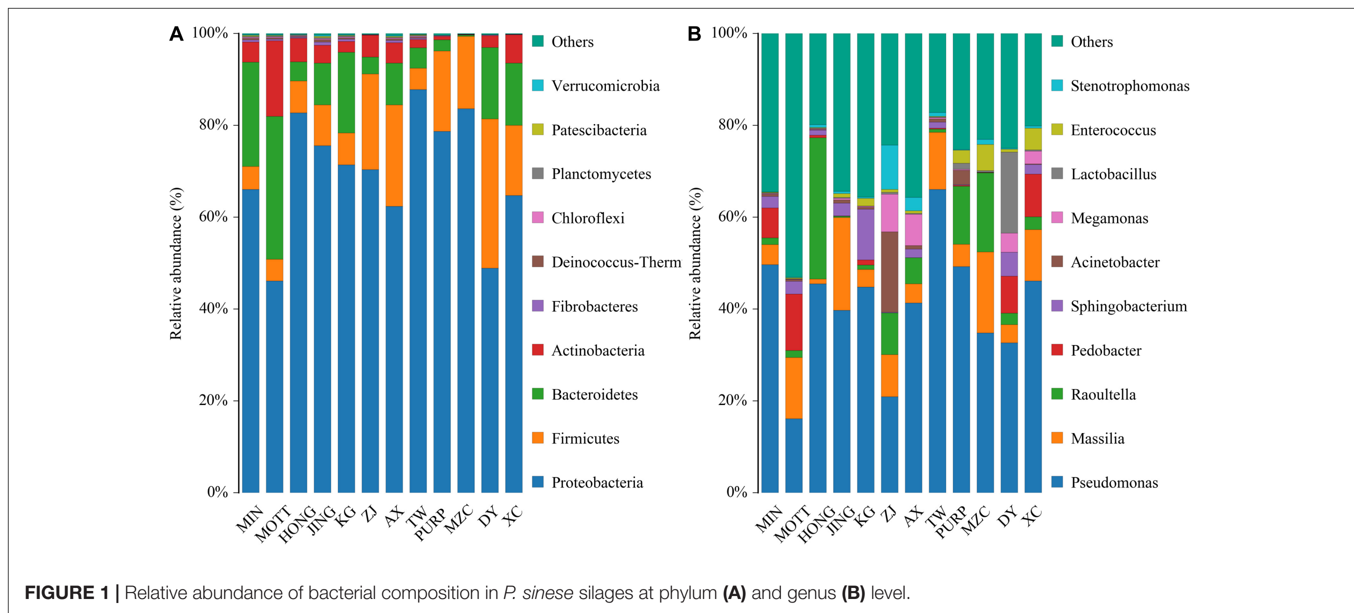

FIGURE 1 | Relative abundance of bacterial composition in *P. sinese* silages at phylum **(A)** and genus **(B)** level.

biomarkers for the silages produced by the different varieties of *P. sinese*.

Association Analysis Between Bacterial Communities and the Chemical Composition of Raw *Pennisetum sinese* Before Ensiling

The correlation between chemical composition (DM, OM, CP, NDF, ADF, and WSC) and the microbiome of *P. sinese* silages was also assessed. At the genus level, a Spearman correlation heat map was created for the microbial communities from the silages (**Figure 3**). *Megamonas*, *Pseudomonas*, *Bacteroides*, *Raoultella*, *Citrobacter*, *Stenotrophomonas*, and *Enterococcus* abundance positively correlated with the DM, OM, CP, NDF, ADF, and WSC content ($P < 0.05$). In addition, *Rhodococcus*, *Chryseobacterium*, *Pedobacter*, and *Verticia* abundance was negatively correlated with these chemical compositions of raw *P. sinese* ($P < 0.05$). This present study showed significant correlation between the species of bacteria and the chemical composition of the raw *P. sinese* used to produce the silage. The results of this study further compound the significant relationship between the chemical composition of the raw material and the resultant silage.

Association Analysis Between Bacterial Communities and Fermentation Characteristics

The correlation between fermentation characteristics and the microbiome of *P. sinese* silages was assessed (**Figure 4**). Silage pH was positively correlated with the abundance of *Bacteroides*, *Megamonas*, and *Citrobacter* ($P < 0.01$) in the silage microbiome, while it was negatively correlated with the abundance of *Chryseobacterium* and *Pedobacter* ($P < 0.01$). The silage lactic acid content was positively correlated with the abundance of *Bacteroides*, *Megamonas*, and *Citrobacter* ($P < 0.01$), while it was negatively correlated with the abundance of *Chryseobacterium*, *Paenibacillus*, *Sphingobacterium*, *Flavobacterium*, *Pedobacter*, and *Rhodococcus* ($P < 0.01$). The acetic acid content of the silage was positively correlated with the abundance of *Citrobacter* ($P < 0.01$), while it was negatively correlated with the abundance of *Chryseobacterium*, *Sphingobacterium*, *Verticia*, *Pedobacter*, and *Rhodococcus* ($P < 0.01$). The propionic acid content of the silage was positively correlated with the abundance of *Bacteroides* ($P < 0.01$), while it was negatively correlated with the abundance of *Chryseobacterium* ($P < 0.01$). Finally, the butyric acid content of the silage was positively correlated with the abundance of *Bacteroides* ($P < 0.01$), while it was negatively correlated with the abundance of *Bradyrhizobium* and *Paenibacillus* ($P < 0.01$).

Association Analysis Between Bacterial Communities and Environmental Factors

The relationship between environmental factors the varying *P. sinese* samples were exposed to **Supplementary Table 1** and the silage they produced was assessed (**Figure 5**). The level of precipitation the *P. sinese* sample was exposed to was positively correlated with the abundance of *Pseudomonas, Stenotrophomonas, Citrobacter, Raoultella, Lactobacillus, Enterococcus*, and *Megamonas* ($P < 0.001$), whilst it was negatively correlated with the abundance of *Rhodococcus, Verticia, Chryseobacterium*, *Pedobacter*, and *Sphingobacterium* ($P < 0.01$). The environmental temperature was positively correlated with the abundance of *Pseudomonas* and *Raoultella* ($P < 0.05$), whilst it was negatively correlated with the abundance of *Rhodococcus, Verticia, Chryseobacterium*, and *Pedobacter* ($P < 0.01$). Meanwhile, environmental humidity was positively correlated with the abundance of *Pseudomonas, Stenotrophomonas*, and *Citrobacter* ($P < 0.01$), whilst it was negatively correlated with the abundance of *Rhodococcus, Verticia* and *Chryseobacterium*, *Pedobacter* ($P < 0.01$). The

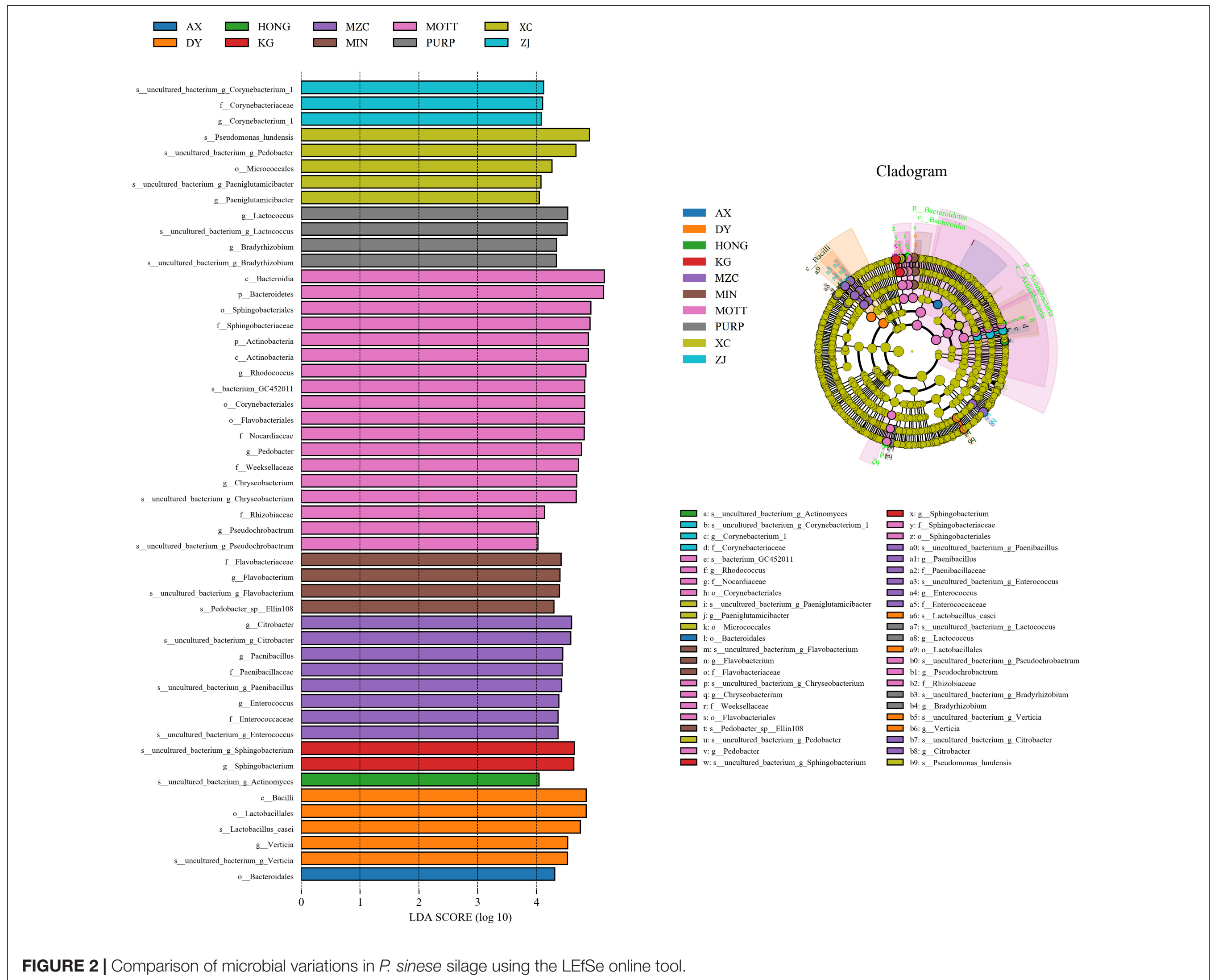

FIGURE 2 | Comparison of microbial variations in *P. sinese* silage using the LEfSe online tool.

longitude and latitude of the base in which the *P. sinese* sample was grown was positively correlated with the abundance of *Pseudomonas, Stenotrophomonas, Citrobacter*, and *Raoultella* ($P < 0.05$), whilst it was negatively correlated with the abundance of *Rhodococcus, Verticia, Chryseobacterium*, and *Pedobacter* ($P < 0.05$). The altitude at which the *P. sinese* sample was grown was positively correlated with the abundance of *Pseudomonas* but no other bacteria, suggesting that altitude played a only a minor role in determining the microbiome content of the silage ($P < 0.05$).

DISCUSSION

Characteristics of *P. sinese* Before Ensiling

The chemical composition of raw materials has an important influence on the silage quality. In this study, the chemical composition of *P. sinese* varieties are similar to a previous study in which king grass was examined (Li et al., 2019b). In contrast, both Mugabe et al. (2019) and Li et al. (2020) reported higher CP, NDF, and ADF content in *P. sinese* varieties compared to the results described. Furthermore, Desta et al. (2016) reported a higher DM, NDF, and ADF content, but lower WSC content, in Napier grass. Shah et al. (2017) also found a higher NDF content, whilst they also found a lower CP and WSC content in king grass compared to the results presented herein. These results imply that there are a large differences in the chemical compositions of different varieties of *P. sinese*; this could influence the silage quality. In particular, low levels of WSC are thought to contribute to the poor fermentation quality of *P. sinese* silage without additives (Wang et al., 2019a).

The Fermentation Properties of *P. sinese* Silage

Silage pH is a very important evaluation parameter to consider when assessing fermentation efficacy; well fermented silage should have a pH of 4.2 or lower (Edwards and McDonald, 1978).

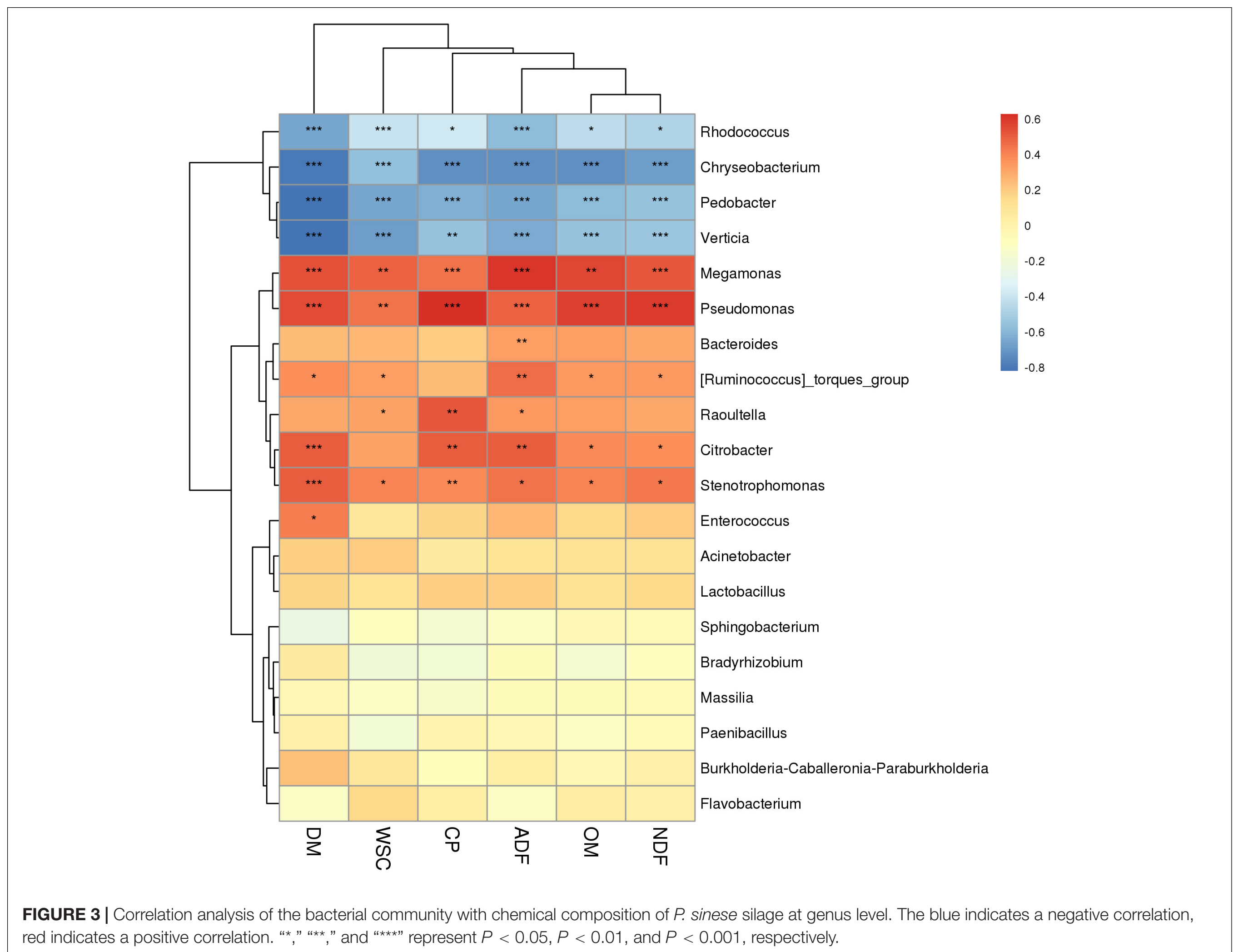

FIGURE 3 | Correlation analysis of the bacterial community with chemical composition of *P. sinese* silage at genus level. The blue indicates a negative correlation, red indicates a positive correlation. "*," "**," and "***" represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

The results presented in this study show that all silages had pH of higher than 4.2, suggesting a lower quality of fermentation. Previous studies have reported a range of silage pH's with some corroborating what was found in this study and other papers showing *P. sinese* silage with a lower pH (Li et al., 2014, 2019a, 2020; Desta et al., 2016; Mugabe et al., 2019). The pH variability may be due to different *P. sinese* varieties being used in this study compared to others, or differences in the silage microbiome. This study revealed relatively low lactic and propionic acid content compared to previous studies. Meanwhile, the acetic acid, butyric acid, and ammonia-N content were similar to, or lower than, the levels reported in previous papers looking at silage produced from *P. sinese* grass (Li et al., 2014, 2019a,b, 2020; Desta et al., 2016; Shah et al., 2017; Mugabe et al., 2019). The differing chemical compositions of the raw *P. sinese* samples may have contributed to the substantial variation in silage fermentation characteristics; Guan et al. (2018) reported this phenomenon previously, albeit in corn silage. Desta et al. (2016) reported the V-Score of Napier grass silage without additive as 63.1, this falls within the range elucidated in this study. All the naturally fermented *P. sinese* silages in this study had a low V-score, which suggests that the silage quality was unsatisfactory. The poor fermentation quality may be due to the low levels of WSC. WSC is an important substrate for lactic acid bacteria (LAB); these are the dominant bacteria in the microbiome of high quality silage; they have been shown to reduce silage pH and prevent the dominance of undesirable microorganisms by producing lactic acid (Guan et al., 2018). The relatively low levels of WSC identified in the silage produced by *P. sinese* varieties in this study may have restricted the growth of LAB, resulting in the production of low quality silage.

However, another important reason for the low silage quality is the higher moisture content of *P. sinese* (77.5∼88.7%). In normal conditions, the ideal moisture content of silage materials is 65–70%, the higher moisture content is conducive to the growth of undesirable microorganisms, which decreased the silage quality. In the tropics of southern China, when wilting grass, there are often showers. In order to avoid rain damage during the silage making, in the present study, we try to prepare *P. sinese* silage with out wilted for investigating their silage quality, microbial community and the correlation with the environmental factor. One caveat with this study, the lower silage

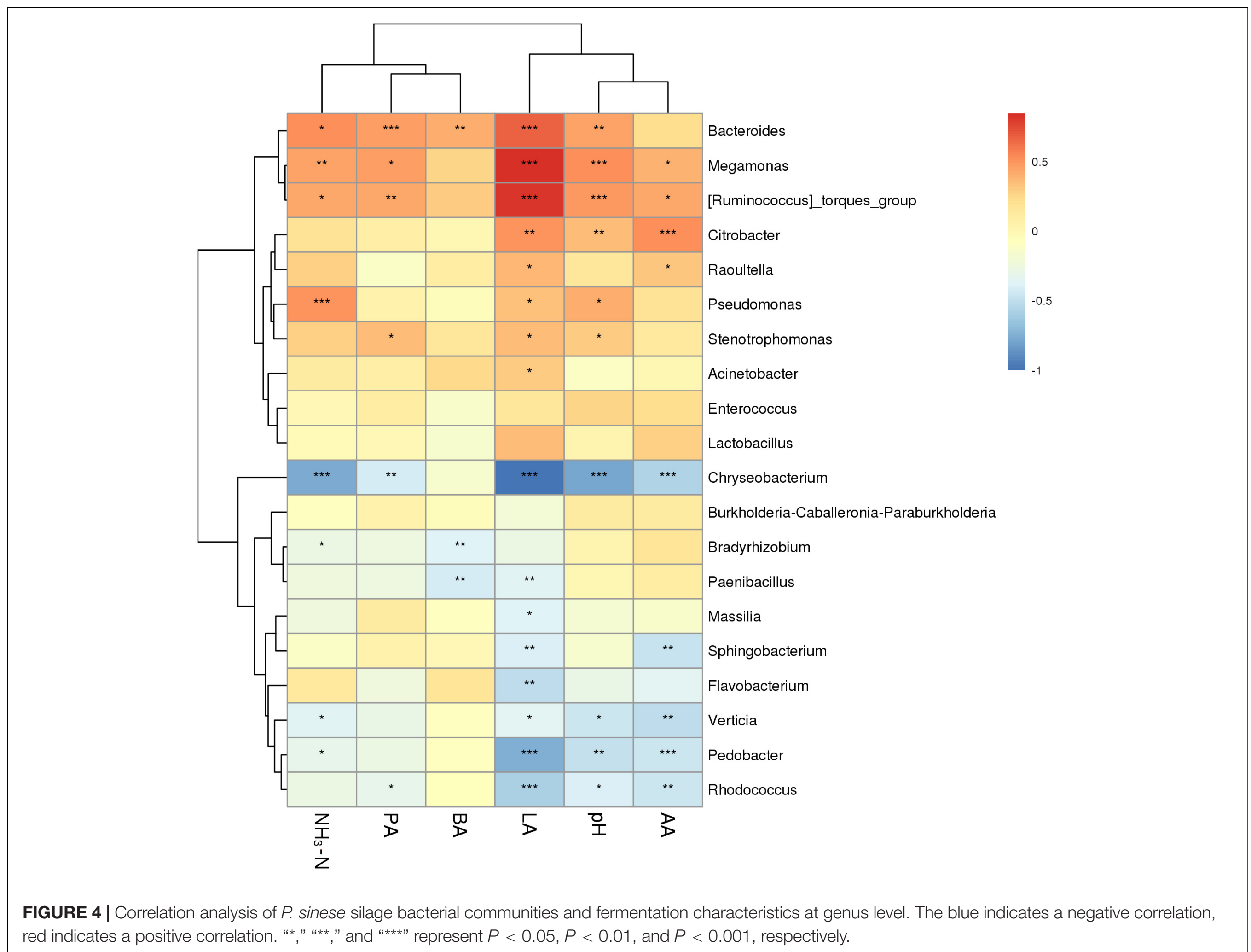

FIGURE 4 | Correlation analysis of *P. sinese* silage bacterial communities and fermentation characteristics at genus level. The blue indicates a negative correlation, red indicates a positive correlation. "*," "**," and "***" represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

quality reminded us ensiled directly was not the suitable way for *P. sinese* preparation.

Bacterial Community of *P. sinese* Silage

In the present study, *Proteobacteria, Firmicutes, Bacteroidetes*, and Actinobacteria were dominant in all *P. sinese* samples. These findings are consistent with previous reports examining *P. sinese*, corn stover and red clover silage (Xu et al., 2017; Dong et al., 2019; Wu et al., 2020) and have been corroborated by previous studies that reported *Firmicutes* and *Proteobacteria* as the most abundant phyla in silages, with their abundance increasing to more than 90% after fermentation (Li L. et al., 2017; Liu et al., 2019; Wang et al., 2019b). Normally, *Lactobacillus* is the major bacterial and dominant genus in well preserved silage, with *Lactobacillus* performing desirable functions during fermentation (Li L. et al., 2017; Liu et al., 2019; Wang et al., 2019b). However, in this study, *Pseudomonas*, *Massilia*, and *Raoultella* were the predominant genera, which is consistent with previous research reporting that these bacteria were associated with the unsatisfactory fermentation of silage (Li L. et al., 2017; Li et al., 2019a; Wu et al., 2020). *Pseudomonas* is considered especially undesirable due to the production of biogenic amines that reduce the protein content and nutritional value of silage (Silla Santos, 1996; Dunière et al., 2013). The present study shows that *Pseudomonas* is one of the most abundant genera of bacteria present in the silages produced by differing *P. sinese* varieties. It is proposed that *Pseudomonas* contributed to the poor *P. sinese* silage quality, as has been previously reported in studies examining forage silage (Xu et al., 2017; Dong et al., 2019; Wang et al., 2019a). However, Ogunade et al. (2018) found that *Pseudomonas* were negatively correlated with pH, ammonium nitrogen, yeast, and mold, suggesting that it may be beneficial to silage fermentation. Therefore, the underlying mechanisms of *Pseudomonas* in silage fermentation processing need to be further investigated.

Correlations Between Bacterial Communities and the Chemical Composition

This study found that there was a significant correlation between the chemical composition of silage raw materials and silage microorganisms. The chemical composition of

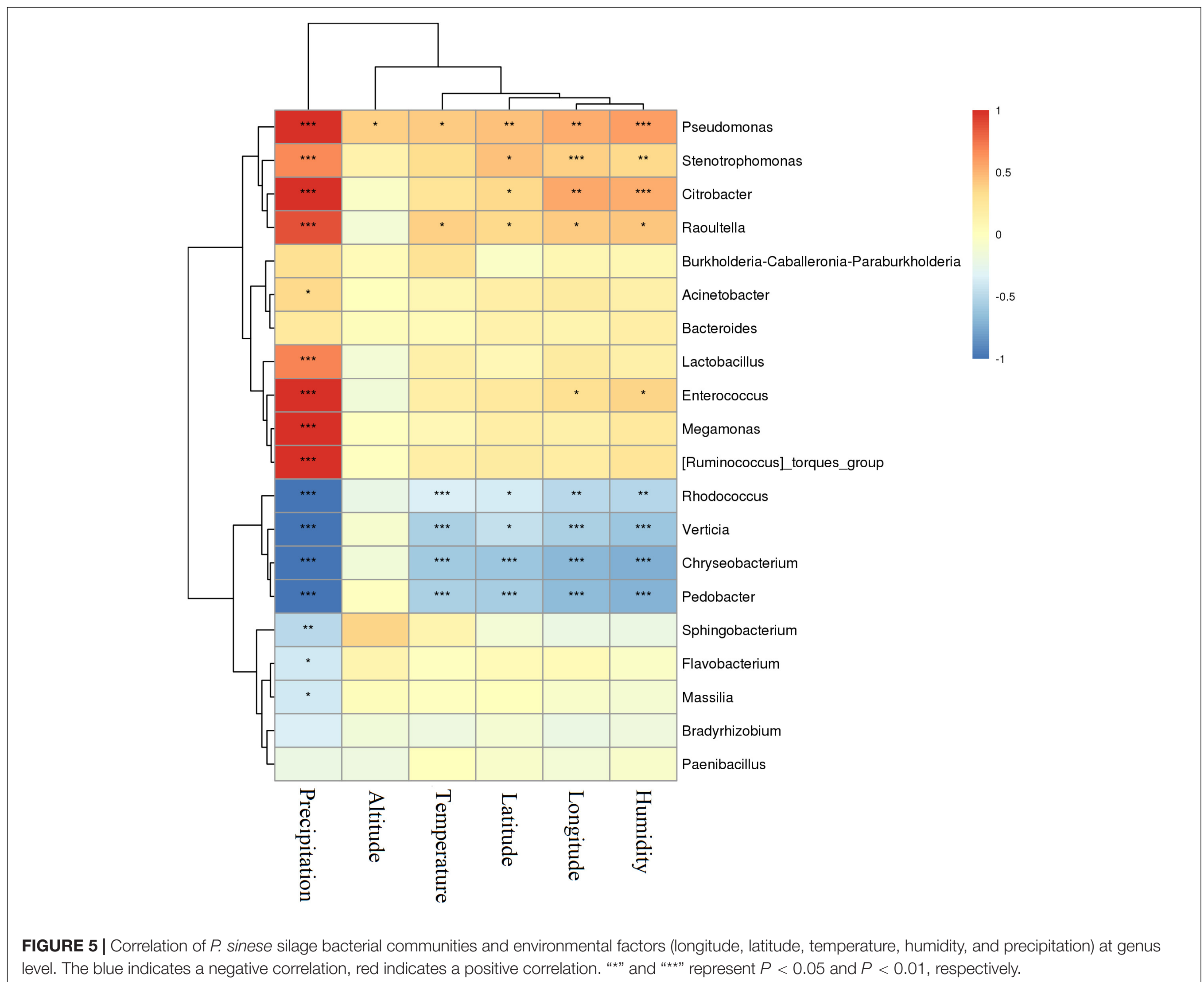

FIGURE 5 | Correlation of *P. sinese* silage bacterial communities and environmental factors (longitude, latitude, temperature, humidity, and precipitation) at genus level. The blue indicates a negative correlation, red indicates a positive correlation. "*" and "**" represent $P < 0.05$ and $P < 0.01$, respectively.

different varieties of *P. sinese* was quite different, so the characteristics of silage microbial community were also different. This may be explained by the fact that these microorganisms are chemoorganotrophic bacteria that produce energy through the oxidation of organic matter such as starch and organic acids. *Pseudomonas*, *Bacteroides*, and *Stenotrophomonas* all consume protein, whilst *Megamonas*, *Raoultella*, *Citrobacter*, and *Enterococcus* ferment carbohydrates (Skerman et al., 1980; Schleifer and Kilpper-Bälz, 1984; Drancourt et al., 2001; Tian et al., 2019) and *Bacteroides* and *Stenotrophomonas* can utilize either (Palleroni and Bradbury, 1993; Silla Santos, 1996; Xu et al., 2003; Dunière et al., 2013; Li M. et al., 2017). Guan et al. (2018) reported that the WSC content of raw corn correlated with the abundance of *Lactobacilli* and *Acetobacter* in the resultant corn silage. Corroboratively, Yang et al. (2019) found the same correlation, however, there was no significant correlation between DM and microorganism abundance in alfalfa silage. The present study showed significant correlation between the species of bacteria and the chemical composition of the raw *P. sinese* used to produce the silage. The results of this study further compound the significant relationship between the chemical composition of the raw material and the resultant silage. Discrepancies between this study and those mentioned above are most likely due to the differences in the chemical composition of the 12 varieties of *P. sinese* and the forage material they used.

Correlations Between Bacterial Communities and Fermentation Characteristics

Silage microorganisms and metabolites are the key factors affecting silage fermentation quality. *Megamonas*, *Bacteroides*, and *Citrobacter* all have the ability to ferment multiple carbohydrates and produce an array of organic acids, including lactic, acetic, and propionic acid (Skerman et al., 1980; Xu et al., 2003; Li M. et al., 2017; Tian et al., 2019). Therefore, it is unsurprising that there was a significant positive correlation between the organic acid content and the microbiome of the

silage. *Pseudomonas* is an undesirable bacteria in silage due to its production of biogenic amines leading to a reduced protein content (Silla Santos, 1996; Dunière et al., 2013). Furthermore, higher levels of ammonium nitrate were associated with increased abundance of *Pseudomonas*. Silage fermentation is a complex biological process which involves a large variety of microorganisms; as such, the process produces many different metabolites which can determine the fermentation quality. Variety in the microbiome of the silage alters the metabolites produced during ensiling and may contribute to the fermentation quality. In the present study, the silage produced from the different varieties of *P. sinese* lacked an abundance of *Lactobacillus*; this may explain why the fermentation quality of the silage was poor. Other studies have corroborated these findings, by showing that fermentation characteristics are highly correlated with the microbiome of the silage, influencing the overall fermentation quality (Guan et al., 2018; Ogunade et al., 2018; Ren et al., 2019; Yang et al., 2019). Therefore, reducing the content of non-lactic acid bacteria is an effective measure to improve the fermentation quality of silage.

Correlations Between Bacterial Communities and Environmental Factors

The results of this study reveal that the microbiome of silage is affected by internal factors (chemical composition of the raw silage material) and external factors (temperature, precipitation levels). This has previously been discussed in other studies, with fermentation metabolites, precipitation, temperature, humidity, longitude, latitude and altitude all being reported to effect the microbiome of the silage and influence the fermentation quality (Muck, 2013; Bernardes et al., 2018; Guan et al., 2018). The results of this study were consistent with previous reports assessing the effect of rainfall and temperature on silage quality (Kim and Adesogan, 2006; Coblentz and Muck, 2012). Guo et al. (2014, 2015) highlighted the importance of sunlight on the fermentation of Napier grass and Italian Rye grass; the results of this study also show that sunlight affects the quality of *P. sinese* silage due to its influence on the silage microbiome. In addition, the storage temperatures of the silage influenced the bacterial diversity/fermentation quality, which had been previously reported in the silage of *Moringa oleifera* leaves (Wang et al., 2019a). Moreover, Guan et al. (2018) reported that environmental factors (temperature, humidity, and precipitation) affected the fermentation quality of silage mediated by changes in the microbiome. These results suggest that high temperature, rain, and high humidity in the south of China have a great negative influence on the microbiome of the silage from *P. sinese*. The reason that environmental factors affect the microbial community of silage may be that environmental differences affect the community structure of epiphytic bacteria on raw materials, which have a greater impact on the initial stage of silage fermentation. With the process of silage fermentation, the impact of environmental factors on the microbial community will be reduced, thus lightened its impact on silage quality. Interestingly, this study identified that longitude and latitude had significant effects on the bacterial diversity of the silage, leading to differences in the fermentation quality of the silage. This suggests that for silage preparation in different regions, geographical factors and their effect on silage microbiome must be considered in order to make provisions to ensure high quality production.

CONCLUSION

The study presented herein examined the microbiome and fermentation quality of silages from the natural fermentation of 12 varieties of *P. sinese* grown across southern China. The silage quality of *P. sinese* was generally unsatisfactory with low V-Scores. There was a predominance of undesirable genera including *Pseudomonas*, *Massilia*, and *Raoultella* in silage produced from *P. sinese;* this led to poor fermentation quality. The strong correlation between the chemical composition of the silage material and the fermentation characteristics and bacterial community of the resultant silage. In addition, precipitation, temperature, humidity, and location significantly influenced the bacterial community of the silage. The specific *P. sinese* cultivar used for silage production and the environmental factors it has been subject to must be considered in order to ensure a high-quality end product.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://catalog.data.gov/dataset/sequence-read-archive-sra, PRJNA624770.

AUTHOR CONTRIBUTIONS

ML, XZ, and DY did the experimental design work. ML, XZ, DY, and JT conducted the experiments. ML, XZ, DY, JT, HZ, and YC analyzed the data. ML and XZ wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.627820/full#supplementary-material

Supplementary Table 1 | Environmental factors effecting the 12 varieties of *P. sinese.* MIN, *Pennisetum purpureum* Schum. cv. Gui Min Yin; TW, *Pennisetum purpureum* Schumab ev. Taiwan; MOTT, *Pennisetum purpureum* cv. Mott; KG, *Pennisetum purpureum* × *P. glaucum* cv. Reyan No. 4; DY, *Pennisetum purpureum* K.; HONG, *Pennisetum purpureum* Schumab cv.; JING, *Pennisetum purpureum* K. Jingmu No. 1; AX, *Pennisetum purpureum* K.; ZJ, *Pennisetum americanum* × *Pennisetum purpureum*; MZC, *Pennisetum glaucum*; PURP, *Pennisetum purpureum* cv. Purple; XC, *Pennisetum purpureum* Schumach.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of Obligate Heterofermentative Lactic Acid Bacteria Alone or in Combination on the Conservation of Sugarcane Silage

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Our objective was to determine the effects of two strains of obligate heterofermentative bacteria, alone or in combination, on the fermentation profile, gas production kinetics, chemical composition, and aerobic stability of sugarcane silage. A plot of sugarcane was manually harvested, mechanically chopped and treated with: distilled water (5 mL kg^{-1}; Control), *Lentilactobacillus hilgardii* CNCM I-4785 [3×10^5 colony-forming units (cfu) g^{-1}; LH], *Lentilactobacillus buchneri* NCIMB 40788 (3×10^5 cfu g^{-1}; LB), and LH+LB (1.5×10^5 cfu g^{-1} of each strain). Treated forages were packed into 1.96-L gas-tight silos (0.40 porosity) and stored at 25 ± 1.5°C for 70 days (4 replicates per treatment). All heterolactic inoculants were effective to increase acetic acid concentration and inhibit yeast metabolism, as treated silages had lower formation of ethanol, ethyl esters and gas during fermentation. Lower fungal development spared soluble carbohydrates, consequently resulting in silages with higher *in vitro* digestibility. Nevertheless, *L. buchneri* was the most effective strain to extend the aerobic stability of sugarcane silage (based on both temperature and pH rise). The use of *L. buchneri* alone or in combination with *L. hilgardii*, applied at 3×10^5 cfu g^{-1}, is a feasible strategy to inhibit yeast metabolism and increase the nutritional quality of sugarcane silage.

Keywords: aerobic stability, acetic acid, gas loss, *Lentilactobacillus buchneri*, *Lentilactobacillus hilgardii*

INTRODUCTION

In tropical areas, sugarcane (*Saccharum officinarum* L.) crop is characterized by a high dry matter (DM) yield (>30 t DM/ha) within one harvest and a suitable nutritive value at maturity (48 h DM digestibility > 600 g kg^{-1}; Daniel et al., 2013b), enabling high animal stocking rates. Although the maximum nutritive value of sugarcane matches pasture shortage during dry season, sugarcane has been ensiled to avoid daily harvesting, prevent crop lodging and prolong field lifespan by better agronomic management (Daniel et al., 2019).

Despite its high fermentation capability (appropriate DM content, low buffering capacity and high content of soluble sugar at ensiling; fermentability coefficient ∼170), sugarcane conserved by natural fermentation has high fermentation loss (up to 300 g kg^{-1} of ensiled DM), due to the formation of ethanol and CO_2 by yeast metabolism (Kung and Stanley, 1982; Pedroso et al., 2005; Ávila et al., 2010). Additionally, high yeast counts in sugarcane silages contribute to aerobic deterioration after feedout, increasing total nutrient losses (Ávila et al., 2012).

A feasible strategy to reduce yeast activity in sugarcane silage, both during storage and feedout phases, is the application of chemical or microbial additives (Pedroso et al., 2011). In general, both chemical additives with antifungal power and obligate heterofermentative lactic acid bacteria (LAB) are effective to protect sugarcane silage against yeast detrimental effects (Daniel et al., 2015b); whereas bacterial inoculants are cheaper than chemical additives (Kung et al., 2003).

Different obligate heterofermentative LAB have been launched in the market during the last two decades (Muck, 2013; Ávila et al., 2014; Daniel et al., 2015a). More recently, the combination of obligate heterofermentative LAB applied at $\geq 3 \times 10^5$ cfu g^{-1} has shown potential benefits in studies with whole-plant corn silage and high moisture corn (Drouin et al., 2019; da Silva et al., 2021; Ferrero et al., 2021). Besides, Ferrero et al. (2021) reported that combination of *L. hilgardii* and *L. buchneri* improved the aerobic stability earlier than that inoculants alone, due to a reduction in yeast population. Whist the effects of single strains of heterolactic bacteria have been reported in different trials, the associative effect of obligate heterofermentative LAB in sugarcane silage is unknown. Additionally, most studies on the effects of obligate heterofermentative LAB in sugarcane silage evaluated relatively low application rates ($\leq 1 \times 10^5$ cfu g^{-1}) compared to doses marketed for different crops in the United States and Europe, which might contribute to controversial benefits of obligate heterofermentative LAB on the conservation of sugarcane silage (Schmidt, 2009; Rabelo et al., 2019).

Thus, the objective of this study was to compare the effectiveness of two strains of obligate heterofermentative LAB [one strain of *Lentilactobacillus buchneri* NCIMB 40788 (isolated from corn silage) and one strain of *Lentilactobacillus hilgardii* CNCM I-4785 (isolated from sugarcane silage)], alone or in combination, on the fermentation traits, microbial counts, fermentation losses, gas production kinetics and nutritional value of sugarcane silage. We hypothesize that either *L. buchneri* and *L. hilgardii* alone or in combination, inoculated at 3×10^5 cfu g^{-1}, would be effective to mitigate yeast detrimental effects in sugarcane silage, but expected that combination of *L. hilgardii* and *L. buchneri* would abate the gas production sooner.

MATERIALS AND METHODS

Ensiling

Sugarcane variety CTC-25 was manually harvested from one plot at the State University of Maringá (Maringá, Brazil) after 10 months of regrowth (2nd cut) and chopped in a stationary cutter to a theoretical cut length of 8 mm. At harvest, sugarcane DM was 307 g kg^{-1} fresh matter (FM) and the content of soluble solids in the stalk juice was 24° Brix, indicating that the crop was mature. Chopped sugarcane was divided in four piles (8 kg fresh matter per pile). Piles were treated with: Control (without additive; 5 mL kg^{-1} of distilled water), *Lentilactobacillus hilgardii* CNCM I-4785 [3×10^5 colony-forming units (cfu) g^{-1}; LH], *Lentilactobacillus buchneri* NCIMB 40788/ CNCM I-4323 (3×10^5 cfu g^{-1}, LB), and LH+LB (1.5×10^5 cfu g^{-1} of *L. hilgardii* + 1.5×10^5 cfu g^{-1} of *L. buchneri*). *L. hilgardii* and *L. buchneri* strains lyophilized and packed in aluminized pouches were donated by Lallemand SAS (Blagnac, France).

Afterward, treated forages were packed (0.40 porosity; Pitt, 1986) into 1.96-L gas-tight silos (∼1,280 g per silo) (Daniel and Nussio, 2015) and stored for 70 days (4 replicates per treatment). Once jar caps sealed, silos were stored in a room with controlled temperature (25 ± 1.5°C).

Gas Production Kinetics and Fermentation Losses

The internal pressure of the silos was measured by using a pressure transducer coupled to a data logger (Daniel and Nussio, 2015). Pressure measures were performed twice daily during the first 3 days of storage, once daily until the end of the third week, every 3 days until the end of 2 months, and then once a week until 70 days. Pressure values were converted to volume (Daniel and Nussio, 2015) and the cumulative gas production per kg of DM was fitted with an exponential 1-pool model [$Gt = (G \times (1 - \exp^{-(k \times t)}))$] to estimate the fractional rate of gas production (k) and gas pool (G) (Daniel et al., 2016). Gas emission was obtained by converting gas volume to CO_2 mass, considering that 1 L of silage gas has 0.99 L of CO_2 and that 1 L of CO_2 has 1.96 g of CO_2 (Daniel and Nussio, 2015). Gas loss also was determined by gravimetry, as the difference of silo mass at ensiling and at opening. Dry matter loss during fermentation was determined by the difference between forage DM mass at ensiling and silage DM mass at opening. Silage DM content used in this calculation was corrected for loss of volatile compounds, as described below.

Laboratory Analysis

At silo opening, silage sub-samples were homogenized with distilled water to prepare aqueous extract (1:10; Kung et al., 1984). Aqueous extracts were used for measuring pH, microbial counts enumerated on selective agar media, and fermentation end-products.

Microbial counts [lactic acid bacteria (LAB), yeasts and molds] were evaluated in a serial dilution of the aqueous extract. Microorganisms were enumerated in Petri dishes with selective media. Malt Extract Agar (M137, Himedia, 632 Mumbai, India) acidified to pH 3.5 with lactic acid was used for enumeration of yeasts and molds, and De Man Rogosa and Sharp (7543A, Acumedia, Lansing, Michigan, United States) supplemented with nystatin (400,000 IU L^{-1}) was used for enumeration of LAB. The plates were incubated aerobically at 30°C for 48 h before counting yeasts and LAB, and for 72 h before counting molds. Colony-forming units (cfu) were counted and expressed as log10.

For analyses of fermentation products, the aqueous extracts were centrifuged at 10,000 × g for 15 min at 4°C. Lactic acid (Pryce, 1969) and NH_3-N (Chaney and Marbach, 1962) were analyzed by colorimetric methods. Volatile fatty acids, alcohols, and esters were determined by gas chromatography-mass spectrometry (GCMS QP 2010 plus, Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax, Restek, Bellefonte, PA; 60 m, 0.25 mm ø, 0.25 μm crossbond carbowax polyethylene glycol). Compounds were identified based on their retention time and mass spectra and quantified with external standards (Lazzari et al., 2021).

Other silage sub-samples were dried at 60°C for 72 h. Dried samples (∼40 g) were ground in a Wiley mill through 1-mm screen and analyzed for absolute dry matter (DMoven; Association of Official Analytical Chemistry [AOAC], 1990; method 934.01), soluble carbohydrates (SC; Hall et al., 1999), crude protein (CP; Association of Official Analytical Chemistry [AOAC], 1990; method 984.13), ash (Association of Official Analytical Chemistry [AOAC], 1990; method 942.05), ether extract (EE; Association of Official Analytical Chemistry [AOAC], 1990; method 945.16), neutral detergent fiber (aNDF; assayed with a heat stable α-amylase and sodium sulfite, and expressed inclusive of residual ash; Mertens, 2002), and acid detergent fiber (ADF; Van Soest, 1963). The content of non-fiber carbohydrate was calculated as follows:

$$\text{NFC}\left(\text{g kg}^{-1}\right) = 100 - (\text{CP} + \text{aNDF} + \text{EE} + \text{ash})$$

The DM_{oven} was corrected for loss of volatile compounds during drying, as follows (Weissbach, 2009):

$$\begin{aligned} &DM_{corr}\ (\text{g kg}^{-1}\text{FM}) \\ &= DM_{oven}\ (\text{g kg}^{-1}\text{FM}) + \text{n-alcohols}\ (\text{g kg}^{-1}\text{FM}) + \\ &\text{2,3-butanediol}\ (\text{g kg}^{-1}\text{FM}) + \text{esters}\ (\text{g kg}^{-1}\text{FM}) + 0.95 \times \\ &\text{volatile fatty acids}\ (\text{g kg}^{-1}\text{FM}) + 0.77 \times \text{1,2-propanediol} \\ &(\text{g kg}^{-1}\text{FM}) + 0.08 \times \text{lactic acid}\ (\text{g kg}^{-1}\text{FM}) \end{aligned}$$

The n-alcohols included methanol, ethanol, and propanol; esters included ethyl lactate, ethyl acetate, and propyl acetate; and volatile fatty acids included acetic, propionic, i-butyric, butyric, i-valeric, and valeric acids.

In vitro DM digestibility was determined using a Daisy II incubator (ANKOM Technology, Macedon, United States). The solutions were prepared according to Tilley and Terry (1963) and the rumen fluid was obtained from two cannulated Holstein cows grazing Bermuda grass, 1 h after supplementation with 2 kg d^{-1} of concentrate based on corn, soybean meal and mineral-vitamin mix.

Aerobic Stability Test

Silage samples (∼1 kg) were loosely placed in plastic jars insulated with polystyrene and exposed to air for 10 days in a room with controlled temperature (25 ± 2°C). Silages were sampled daily (10 g d^{-1}) for measuring pH. Samples were collected without disturbing the silage mass, at approximately 10 cm deep. Temperature was recorded automatically every 15 min with a data logger inserted in the mass at approximately 15 cm deep. Simultaneously, two data loggers recorded the ambient temperature. Aerobic stability was denoted as the length of time that elapsed before silage and ambient temperatures differed by more than 2°C (O'Kiely, 1993).

Statistical Analysis

Data were analyzed using the Mixed procedure of SAS (v. 9.4, SAS Institute Inc., Cary, NC), as a completely randomized design. Silage pH during aerobic exposure was analyzed as repeated measures, including the effect of day of exposure in the previous model. The effect of silo nested with treatment was used as error term. A first-order autoregressive covariance structure was defined because it resulted in the lowest corrected Akaike information criterion. Means were compared by Tukey-Kramer test ($\alpha = 0.05$). Pearson correlations between acetic acid concentration and yeast count, ethanol concentration, gas production, DM loss, and aerobic stability were stablished using the Corr procedure of SAS.

RESULTS

Silage treated with LB and LH+LB had higher ($P < 0.01$) DM_{oven} and DM_{corr} compared to LH and control silages (**Table 1**). Silage pH at opening was relatively low in all treatments, but LH alone or associated with LB led to lowest pH ($P < 0.01$), whereas LB had an intermediate value. Overall, obligate heterofermentative LAB resulted in silages with higher contents of acetic acid, 2,3-butanediol and lower contents of ethanol, methanol, 1-propanol, and ethyl lactate compared to control ($P < 0.01$). The LB and LB+LH silages had higher concentrations of 1,2-propanediol than LH and control silages ($P < 0.01$). Ethyl acetate concentration decreased in treated silages compared to control, but LB and LB+LH silages had lowest concentrations ($P < 0.01$). Ammonia-N, lactic acid, propionic acid, butyric acid, i-butyric acid, i-valeric acid, valeric acid and mold counts were similar among treatments ($P \geq 0.11$). The LH had lower counts of LAB than LB or untreated silage, whereas LH+LB showed intermediate counts of LAB ($P < 0.01$). Yeast counts were reduced in silages inoculated with heterolactic strains, but LH had slightly higher count of yeasts than silage inoculated with *L. buchneri* alone or in combination with *L. hilgardii* ($P < 0.01$). Acetic acid concentration was negatively correlated with yeast count ($r = -0.76$), ethanol concentration ($r = -0.94$), gas production ($r = -0.96$), and DM loss ($r = -0.87$), and positively correlated with aerobic stability ($r = 0.60$) ($P < 0.01$).

Compared with untreated silage, inoculation with obligate heterofermentative LAB consistently reduced ($P < 0.01$) gas and DM losses during fermentation (**Table 2** and **Figure 1**). Compared to untreated silage, aerobic stability based on temperature rise was increased by the inoculation with *L. buchneri* ($P = 0.02$). Silage pH pattern during aerobic exposure indicated that LB and LH+LB delayed pH rise ($P < 0.01$) (**Figure 2**).

Silages treated with obligate heterotactic bacteria presented lower contents of EE ($P = 0.02$), CP, aNDF and ADF ($P < 0.01$), and higher contents of NFC and SC ($P < 0.01$), as well as greater *in vitro* DM digestibility ($P < 0.01$) (**Table 3**).

DISCUSSION

Mature sugarcane is recognized by its high SC concentration at harvest (>350–400 g kg^{-1} of sucrose on DM basis; Cabezas-Garcia et al., 2011); but ensiling sugarcane can decrease its nutritive value due to the consumption of SC, mainly by yeast metabolism (Pedroso et al., 2005; Ávila et al., 2010). Anaerobically, yeasts can convert a large proportion of SC to ethanol and CO_2 which results in high DM loss, while ethanol does not contribute to silage conservation (McDonald et al., 1991). Although ethanol has a higher content of gross energy than carbohydrates (29.7 vs. 17.6 kJ g^{-1}), replacing carbohydrates with ethanol has led to similar or even lower feed efficiency, without effecting DM intake (Randby et al., 1999; Daniel et al., 2013a). Ethanol is partially volatilized from silage (Daniel et al., 2013c; Hafner et al., 2013) and partially converted to acetate and methane in the rumen (Yoshii et al., 2005), decreasing the recovery of net energy (Daniel and Nussio, 2011). Hence, alcoholic fermentation is undesirable and the use of silage additives capable of inhibiting fungi metabolism is required to obtain sugarcane silages with suitable nutritive value (Pedroso et al., 2006). In the current study we showed that applying obligate heterofermentative LAB, alone or in combination, consistently improved sugarcane silage preservation, by increasing acetic acid, inhibiting yeasts, decreasing ethanol and gas loss, sparing SC and improving *in vitro* digestibility.

The strains tested in our study are recognized by their capacity to increase acetic acid concentration in different silage types (Kleinschmit and Kung, 2006; Ávila et al., 2014; Drouin et al., 2019; Gomes et al., 2019; da Silva et al., 2021; Ferrero et al., 2021), by producing lactic and acetic acids from SC (Kandler, 1983; McDonald et al., 1991; Pahlow et al., 2003) or converting lactic acid to acetic acid and 1,2-propanediol (Oude Elferink et al., 2001). Usually, a lower lactic acid concentration is likely expected in silages treated with obligatory heterofermentative LAB (Kleinschmit and Kung, 2006). In our study, acetic acid concentration was increased by 81% (on average) in silages treated with obligate heterofermentative LAB compared to control, whereas the concentration of lactic acid was not modified by the inoculants. It may suggest that lactic acid may have been produced in a lesser extent at the onset of fermentation in control silage whereas in treated silages lactic acid was partially converted to acetic acid and 1,2-propanediol. In fact, control silage had a higher pH value compared with inoculated silages and, although acetic acid has a greater pka than lactic acid (4.8 vs. 3.8), the greater concentration of acetic acid in treated silages might have contributed to pH drop, particularly due to the low buffering capacity in sugarcane crop (Custódio et al., 2016).

Contrary to previous studies that evaluated *L. hilgardii* in sugarcane silage (e.g., Carvalho et al., 2015), corn silage (e.g., Drouin et al., 2019; Ferrero et al., 2021) or high moisture corn (e.g., da Silva et al., 2021), in this work only silage inoculated with *L. buchneri* had significant concentration of 1,2-propanediol. It strongly suggests that *L. hilgardii* produced most acetic acid by fermenting sugars (SC → lactic acid + acetic acid)

TABLE 1 | Fermentation profile and microbial counts in sugarcane silage treated with heterolactic inoculants and stored for 70 days.

Item	Control	LH	LB	LH+LB	SEM	*P*-value
DM_{oven} (g kg^{-1} as fed)	213^c	264^b	284^a	289^a	3.80	<0.01
DM_{corr} (g kg^{-1} as fed)	253^c	285^b	303^a	307^a	3.7	<0.01
pH	3.57^a	3.46^c	3.48^b	3.45^c	0.006	<0.01
NH_3-N (g kg^{-1} N)	60.4	62.2	65.5	62.3	2.28	0.48
Lactic acid (g kg^{-1} DM_{corr})	29.2	30.7	29.9	29.0	2.09	0.94
Ethanol (g kg^{-1} DM_{corr})	126^a	15.6^b	10.5^b	7.55^b	3.83	<0.01
Acetic acid (g kg^{-1} DM_{corr})	21.6^b	39.2^a	38.4^a	39.9^a	1.39	<0.01
2,3-Butanediol (g kg^{-1} DM_{corr})	4.15^b	9.35^a	9.03^a	9.15^a	0.439	<0.01
Ethyl lactate (mg kg^{-1} DM_{corr})	1786^a	490^b	347^b	241^b	98.1	<0.01
Ethyl acetate (mg kg^{-1} DM_{corr})	550^a	298^b	142^c	152^c	32.9	<0.01
1,2-Propanediol (mg kg^{-1} DM_{corr})	192^c	219^c	1642^a	754^b	92.8	<0.01
Propionic acid (mg kg^{-1} DM_{corr})	297	299	314	269	18.6	0.43
Methanol (mg kg^{-1} DM_{corr})	50^a	32^b	30^b	30^b	2.1	<0.01
Butyric acid (mg kg^{-1} DM_{corr})	14	13	9	10	1.7	0.16
1-Propanol (mg kg^{-1} DM_{corr})	14^a	4^b	3^b	4^b	0.6	<0.01
i-Butyric acid (mg kg^{-1} DM_{corr})	3	6	4	5	0.7	0.11
i-Valeric acid (mg kg^{-1} DM_{corr})	6	6	5	4	0.8	0.62
Valeric acid (mg kg^{-1} DM_{corr})	6	6	5	4	0.8	0.31
Lactic acid bacteria (log cfu g^{-1})	4.87^a	2.65^b	4.84^a	3.50^{ab}	0.286	<0.01
Molds (log cfu g^{-1})	<2	<2	<2	<2	–	–
Yeasts (log cfu g^{-1})	4.42^a	2.06^b	$<2^c$	$<2^c$	0.459	<0.01

Control, without additive; LH, Lactobacillus hilgardii CNCM I-4785 at 3 × 10^5 cfu g^{-1}; LB, L. buchneri NCIMB 40788 at 3 × 10^5 cfu g^{-1}; LH+LB, Lactobacillus hilgardii CNCM I-4785 at 1.5 × 10^5 cfu g^{-1} + L. buchneri NCIMB 40788 at 1.5 × 10^5 cfu g^{-1}; SEM, standard error of the mean.
[a,b,c]*Means within a row with different superscripts differ (Tukey test, α = 0.05).*

TABLE 2 | Fermentation losses and aerobic stability of sugarcane silage treated with heterolactic inoculants and stored for 70 days.

Item	Control	LH	LB	LH+LB	SEM	*P*-value
DM_{oven} loss (g kg^{-1} DM)	335^a	153^b	89.2^c	69.5^c	11.94	<0.01
DM_{corr} loss (g kg^{-1} DM)	176^a	56.0^b	47.4^b	46.2^b	2.66	<0.01
Gas loss (g kg^{-1} DM)	129^a	46.0^b	43.9^b	41.3^b	2.20	<0.01
Gas emission[d] (g kg^{-1} DM)	130^a	45.9^b	44.6^b	40.8^b	1.73	<0.01
Gas pool (mL kg^{-1} DM)	73.8^a	23.1^b	22.7^b	20.6^b	1.35	<0.01
Fractional rate of gas production (d^{-1})	0.040^b	0.169^a	0.167^a	0.177^a	0.0064	<0.01
Aerobic stability[e] (h)	144^b	143^b	163^a	158^{ab}	4.51	0.02

Control, without additive; LH, Lactobacillus hilgardii CNCM I-4785 at 3 × 10^5 cfu/g; LB, L. buchneri NCIMB 40788 at 3 × 10^5 cfu g^{-1}; LH+LB, Lactobacillus hilgardii CNCM I-4785 at 1.5 × 10^5 cfu g^{-1} + L. buchneri NCIMB 40788 at 1.5 × 10^5 cfu g^{-1}; SEM, standard error of the mean.
[a,b,c]*Means within a row with different superscripts differ (Tukey test, α = 0.05).*
[d]*Estimated from gas volume.*
[e]*Based on temperature rise.*

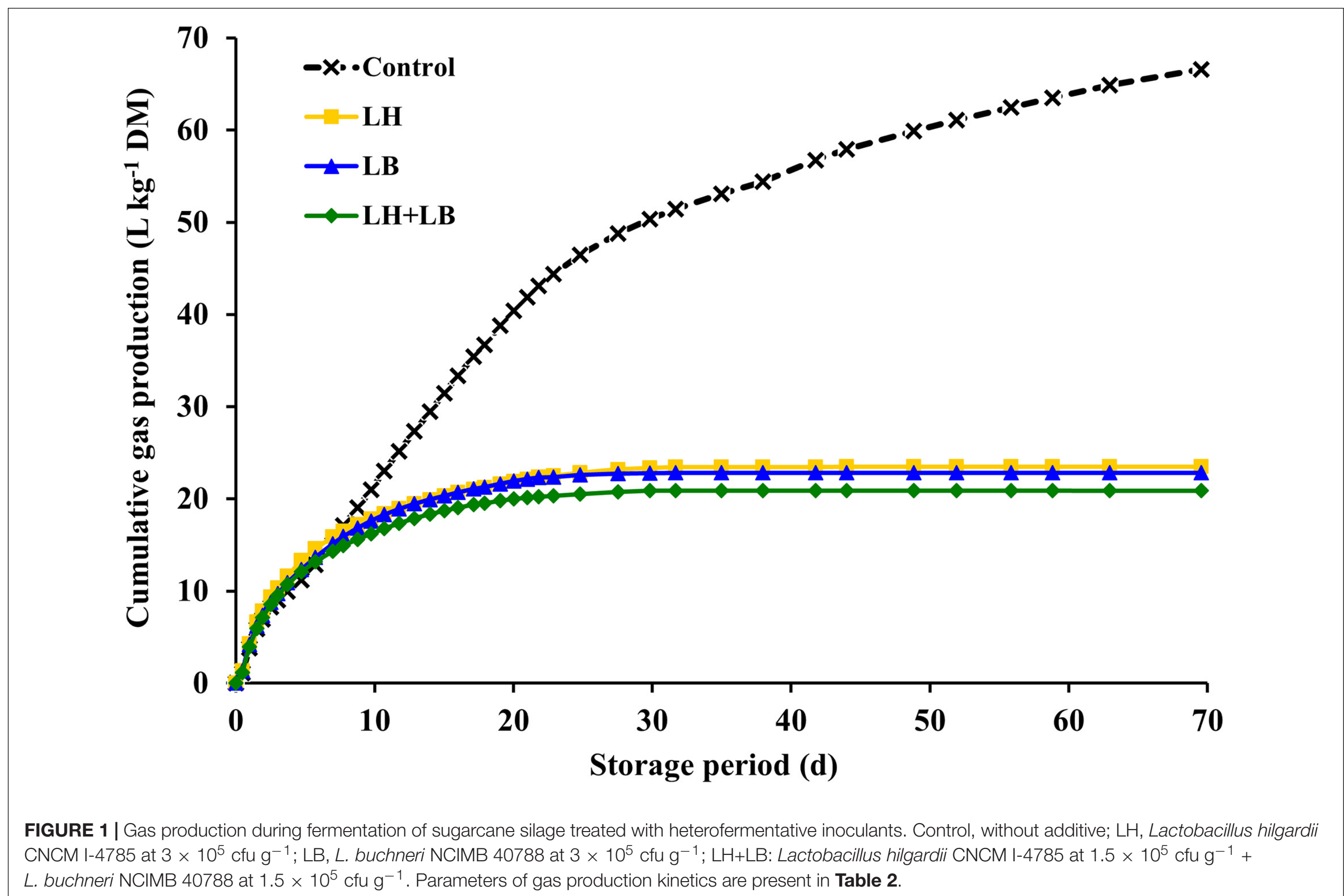

FIGURE 1 | Gas production during fermentation of sugarcane silage treated with heterofermentative inoculants. Control, without additive; LH, *Lactobacillus hilgardii* CNCM I-4785 at 3 × 10^5 cfu g^{-1}; LB, *L. buchneri* NCIMB 40788 at 3 × 10^5 cfu g^{-1}; LH+LB: *Lactobacillus hilgardii* CNCM I-4785 at 1.5 × 10^5 cfu g^{-1} + *L. buchneri* NCIMB 40788 at 1.5 × 10^5 cfu g^{-1}. Parameters of gas production kinetics are present in **Table 2**.

whereas *L. buchneri* converted lactic acid to acetic acid and 1,2-propanediol. The concentration of 1,2-propanediol in LH silage was similar to the control and the combination LH+LB (half dose of each strain) resulted in a concentration of 1,2-propanediol that approach half of that concentration found in silage treated with a full dose of LB. Meanwhile, the content of acetic acid in LB exceeding that in control is close to the content of 1,2-propanediol in LB exceeding that in control, which support the ratio of approximately 0.5 mol of acetic acid to 0.5 mol of 1,2-propanediol converted from 1 mol of lactic acid reported by Oude Elferink et al. (2001). Hence, it seems that *L. buchneri* is more dependent on lactic acid preformed (during fermentation) as substrate whereas *L. hilgardii* prefers sugars to produce acetic acid, at least under the current conditions of strict anaerobiosis (gas-tight silos) with surplus of SC.

Acetic acid is a powerful antifungal compound capable of inhibit yeast metabolism during fermentation (anaerobiosis) and after feedout (aerobiosis) (Moon, 1983; Danner et al., 2003). In our study, acetic acid concentration was negatively correlated with yeast count, ethanol concentration, gas production and DM loss, and positively correlated with aerobic stability. In cereal crops (e.g., corn silage and small grain silages) inoculation with obligate heterofermentative LAB usually resulted in greater DM loss during fermentation by <1–6%-unit (Weinberg and Muck, 1996; Kleinschmit and Kung, 2006; Gomes et al., 2019). However, in sugarcane silages the inoculation with obligate heterofermentative LAB decreases gas loss during fermentation, because of the total amount of CO_2 released as a byproduct of acetic acid formation is by far lower than the amount of CO_2 released during ethanolic fermentation. In this study, obligate heterofermentative LAB not only decreased gas loss or gas emission by 66% but also curtailed the period of gas production. In control silage, 90% of gas pool was achieved at 58 days of fermentation whereas in inoculated silages that was achieved in 2 weeks (13 days). It means that after 2 weeks, the obligate heterofermentative LAB were very effective to inhibit yeast anaerobic metabolism in sugarcane silage.

As expected, the formation of low molecular weight esters was diminished in treated silages, due to the fact that concentration of ethyl esters is positively correlated with ethanol concentration (Hafner et al., 2014; Weiss, 2017). Under acidic conditions, ethanol condenses with acetic acid generating ethyl acetate, whereas ethyl lactate is formed by the esterification of ethanol on lactic acid (Hangx et al., 2001). In this way, the concentration of ethyl acetate was numerically higher in LH than in LB or LH+LB, certainly because the numerically higher concentration of ethanol in LH silage. Although there is a claim that low molecular weight esters could impair the voluntary feed intake (Weiss et al., 2016; Gerlach et al., 2019) reported no negative effects of ethyl esters (ethyl acetate and ethyl lactate) on DM intake and feed preference

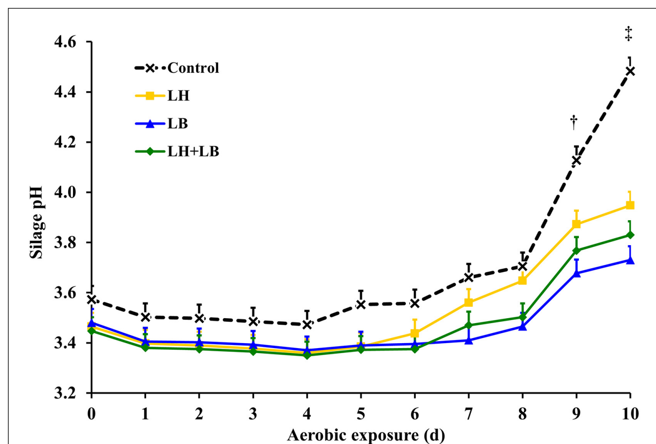

FIGURE 2 | Silage pH during aerobic exposure, after 70 days of storage. Control, without additive; LH, *Lactobacillus hilgardii* CNCM I-4785 at 3×10^5 cfu g^{-1}; LB, *L. buchneri* NCIMB 40788 at 3×10^5 cfu g^{-1}; LH+LB, *Lactobacillus hilgardii* CNCM I-4785 at 1.5×10^5 cfu g^{-1} + *L. buchneri* NCIMB 40788 at 1.5×10^5 cfu g^{-1}. $P < 0.01$ for treatment effect, $P < 001$ for day effect, and $P < 0.01$ for interaction treatment × day. Pooled standard error of the mean = 0.061. $^{\dagger}P < 0.05$ for Control vs. LB and Control vs. LH+LB on day 9. $^{\ddagger}P < 0.05$ for Control vs. LB, Control vs. LH and Control vs. LH+LB on day 10.

TABLE 3 | Chemical composition and *in vitro* digestibility of sugarcane silage treated with heterolactic inoculants and stored for 70 days.

Item	Control	LH	LB	LH+LB	SEM	*P*-value
Crude protein (g kg^{-1} DM_{corr})	25.8^a	22.3^b	20.9^b	20.7^b	0.69	<0.01
aNDF (g kg^{-1} DM_{corr})	661^a	521^b	497^b	499^b	10.8	<0.01
ADF (g kg^{-1} DM_{corr})	403^a	306^b	296^b	297^b	7.6	<0.01
Ash (g kg^{-1} DM_{corr})	35.6^a	29.3^b	27.5^b	27.3^b	0.76	<0.01
Ether extract (g kg^{-1} DM_{corr})	12.4^a	9.48^b	10.1^b	10.4^b	0.59	0.02
Non-fiber carbohydrates (g kg^{-1} DM_{corr})	266^b	418^a	445^a	443^a	11.1	<0.01
Soluble carbohydrates (g kg^{-1} DM_{corr})	213^b	355^a	378^a	374^a	9.28	<0.01
In vitro DM digestibility (g kg^{-1} DM_{corr})	535^b	619^a	629^a	629^a	7.48	<0.01

Control, without additive; LH, Lactobacillus hilgardii CNCM I-4785 at 3×10^5 cfu/g; LB, L. buchneri NCIMB 40788 at 3×10^5 cfu g^{-1}; LH+LB, Lactobacillus hilgardii CNCM I-4785 at 1.5×10^5 cfu g^{-1} + L. buchneri NCIMB 40788 at 1.5×10^5 cfu g^{-1}; SEM, standard error of the mean.
^{a,b}*Means within a row with different superscripts differ (Tukey test, α = 0.05).*

by goats. Meanwhile, esters are highly volatile and associated with poorer air condition in farms feeding silage-based total mixed rations (Hafner et al., 2013).

Although found at very low concentrations, methanol and 1-propanol were decreased in silages treated with obligate heterofermentative LAB. In silages, methanol is likely a product of pectin demethylation (Steidlová and Kalac, 2002; Parra et al., 2019), due to the activity of pectinolytic enzymes, which are widely distributed in nature and are produced by yeast, bacteria, fungi and plants (Sieiro et al., 2012). Dato et al. (2005) reported that contaminating yeasts produced methanol during fermentation of sugarcane juice to produce *cachaça* (an alcoholic beverage), which has a fermentation pattern relatively similar to that in sugarcane silage. Therefore, a higher yeast metabolism might have increased pectin demethylation and methanol formation in control silage.

1-Propanol in silage can be produced by yeasts (McDonald et al., 1991; Giudici et al., 1993), clostridia (Janssen, 2004) and LAB such as *Lentilactobacillus diolivorans* (basonym *Lactobacillus diolivorans*) capable of converting 1,2-propanediol to propionic acid and 1-propanol (Krooneman et al., 2002). It is unlikely that *L. diolivorans* had developed substantially in this study, as our silages had low concentrations of propionic acid and 1-propanol, even in that cases where 1,2-propanediol was abundant. Also, low concentration of butyric acid (<14 mg kg^{-1} DM_{corr}) indicated that *Clostridium* sp. was suppressed during sugarcane silage fermentation. Therefore, 1-propanol might have been derived of yeast activity, as greater concentration of 1-propanol was observed in untreated silage.

Interestingly, all inoculated silages presented more 2,3-butanediol than control. The 2,3-butanediol can be produced by different microorganisms, such as LAB and bacillus (McDonald et al., 1991), clostridia (Siemerink et al., 2011; Fernandes et al., 2020), and enterobacteria (Rooke and Hatfield, 2003; Nishino and Shinde, 2007). Recently, Gomes et al. (2019) reported a consistently higher concentration of 2,3-butanediol in oat silages inoculated with *L. buchneri*. They attributed that greater formation of 2,3-butanediol to enterobacteria (a group of bacteria that develop under non-acidic conditions), because of higher silage pH and silage heterogeneity (Pahlow et al., 2003), due to the possible formation of ecological niches with higher pH in silages treated with *L. buchneri*. In the current study, the origin of 2,3-butanediol is less clear, because treated silages had lower pH than control. Additionally, a low conversion of lactic acid to acetic acid and 1,2-propanediol in the LH treatment (discussed above) weakens the hypothesis of ecological niches that would promote enterobacteria development (Pahlow et al., 2003). Further studies are warranted to explain why 2,3-butanediol may increase in silage inoculated with obligate heterofermentative LAB.

In sugarcane silages, aerobic deterioration is often initiated by lactate-utilizing yeasts, which result in silage heating and pH rise, enabling the proliferation of fungi and aerobic bacteria less tolerant to acidity (Ávila et al., 2012). Acetic acid bacteria can also be involved in aerobic deterioration of sugarcane silage (Daniel, unpublished). In the current trial, a slight decay of silage pH after 24 h of aerobic exposure suggests that ethanol might have been partially oxidized to acetic acid (Spoelstra et al., 1988).

Meanwhile, the rate of aerobic deterioration is driven by a combination of factors such as the initial counts of spoiling microorganisms, substrate availability (mainly SC and lactic acid) and presence of inhibitory substances, such as acetic acid (Danner et al., 2003). In the current study, *L. buchneri* and *L. hilgardii* strains increased acetic acid concentration and spared SC in a similar degree, but *L. buchneri* was the most effective strain to improve aerobic stability, based on a collective interpretation of temperature and pH rise during the aerobic stability test. On the other hand, the time elapsed for temperature rise in

LH silage was similar to that in control, probably due to the greater yeast population present in LH treatment compared with *L. buchneri*. Hence, the combination of LH+LB (half dose of each strain) resulted in intermediate values of aerobic stability based on temperature rise. Collectively, those results support the idea that silage aerobic stability is not only a function of acetic acid concentration or substrate availability for aerobic microorganisms. Also, the fungal communities may have shifted from lactate to non-lactate utilizing yeasts, as recently reported by da Silva et al. (2020) in corn silage treated with chemical additives.

Our results of aerobic stability for the LH treatment contrasted with those reported by Ávila et al. (2014) and Carvalho et al. (2015) who described longer stability in sugarcane silages treated with *L. hilgardii* compared to untreated silage. However, Rabelo et al. (2019) demonstrated by meta-analysis that the effect of obligate heterofermentative LAB on the aerobic stability of sugarcane silages is not consistent. In our study, sugarcane silages (including the control) showed an unusual prolonged aerobic stability compared with previous reports. The aerobic stability of sugarcane silages in studies compiled by Rabelo et al. (2019) ranged from 30 to 100 h. The longer aerobic stability reached in our study might be due to the strictly anaerobic conditions imposed by the gas-tight silos. In fact, yeast counts were lower than 5 log cfu g^{-1} in all treatments, which is considered as threshold count for promptly aerobic deterioration (Borreani et al., 2018).

All nutrient changes observed among treatments were consequence of the consumption or sparing of SC. In treated silages, the microbial inoculants spared SC, whereas in control silage high yeast activity consumed 42% more SC in comparison with inoculated silages, increasing the concentrations of fiber (i.e., aNDF and ADF) and non-carbohydrate nutrients (i.e., CP, ash and EE) by a dilution effect. As the true digestibility of the organic matter soluble in neutral detergent solution (i.e., NFC, CP and EE) is almost complete (Van Soest, 1967; Daniel et al., 2017), DM digestibility is primarily a function of the concentration and digestibility of aNDF. The sugarcane crop used in this study was harvested from the same field and obviously had similar aNDF digestibility among treatments. Therefore, silages with lower aNDF were highly expected to have the greater observed *in vitro* DM digestibility. Other studies evaluating obligate heterofermentative LAB in sugarcane silage have been less consist, with higher (Andrade et al., 2016), equal (Pedroso et al., 2007, 2008) or even lower DM digestibility in treated silage compared with that in control (Rabelo et al., 2019). Differences of inoculant dose among studies (often lower than used in the current study) might partially explain those divergent findings.

CONCLUSION

All heterolactic inoculants applied at 3×10^5 cfu g^{-1} were effective to inhibit yeast metabolism and mitigate gas loss during fermentation resulting in sugarcane silages with greater nutritional value. Nevertheless, *L. buchneri* strain 40788 was the most effective strain to extend the aerobic stability of sugarcane silage in addition to the improved nutrient recovery during fermentation. Our findings do not support the requirement of crop specific strains of obligate heterofermentative LAB to improve the conservation of sugarcane silage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this manuscript.

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Effects of Gallic Acid on Fermentation Parameters, Protein Fraction, and Bacterial Community of Whole Plant Soybean Silage

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Whole plant soybean (WPS) is a kind of legume resource with characteristics of high nutrition, large biomass, and wide distribution. In the present study, we have investigated the feasibility and effects of gallic acid (GA) on WPS silage quality, nitrogen distribution, tannin content, and bacterial community. The 0.5 and 1% (fresh matter basis) GA were added into WPS for dynamic ensiling (days 3, 7, 14, and 30, respectively). The results showed that the WPS silage with GA addition significantly decreased pH value (6.16–5.38 at ensiling day 30), coliform bacteria count and butyric acid (65.3–62.0 g/kg dry matter at ensiling day 30), and amino nitrogen contents (259–88.2 g/kg total nitrogen at ensiling day 30) and promoted lactic acid (9.62–31.5 g/kg dry matter at ensiling day 30), acetic acid (24.1–85.6 g/kg dry matter at ensiling day 30), and tannin (total phenol and hydrolyzable tannin) contents. Additionally, the GA addition also contributed to the change of bacterial community, where *Firmicutes* and *Lactobacillus* were most abundant on phylum and genus levels, respectively. The above results suggested that GA additive applied in WPS silage was an effective strategy to protect nutrition and improve fermentation quality, and the 1% GA addition showed a better effect.

Keywords: whole plant soybean, fermentation quality, protein fraction, bacterial community, gallic acid

INTRODUCTION

Soybean (*Glycine max* L. Merrill), one of the most valuable oilseed crops all over the world (Dubey et al., 2018), and its by-product soymeal are unarguably considered the most economical plant proteins, thus are commonly used in animal diets. Whole plant soybean (WPS), including stems, leaves, and fruits, is also nutritious and easy to collect and operate during maturity period. However, WPS is mainly directly used as an inefficient animal feed or a rural fuel or is returned to the field or even arbitrarily discarded in recent years (Liu et al., 2015). Thus, optimizing modulation technology is a crucial step for rational utilization of WPS resources. Ensiling is commonly used to store forage biomass, with the characteristics of easy operation, low cost, and less nutrition loss

(Liu et al., 2011). But as the legume, WPS might be difficult to achieve a satisfactory silage quality. That is mainly because of the low water-soluble carbohydrates (WSCs) and insufficient epiphytic lactic acid bacteria (LAB) count of fresh WPS, as WSC contents need higher than 60 g/kg dry matter and epiphytic LAB count not below 5 $\log_{10}$ CFU/g fresh matter (FM) for obtaining well-preserved silage (Yang et al., 2016). Otherwise, the silage pH cannot decline rapidly, and undesirable microorganisms like *Clostridia* and *Enterobacter* lead to butyric acid production and proteolysis during ensiling (Silva et al., 2016). Therefore, it is needed to quest some strategies to decelerate even prevent these adverse effects for the purpose of protecting forage nitrogen and improving WPS silage quality (Anuraga et al., 2018).

Gallic acid (GA), with the chemical structure of three phenolic hydroxyl groups and one carboxyl group, is also called 3,4,5-trihydroxybenzoic acid or gallo-tannin (Bharti et al., 2015). Generally, GA exhibits a broad range of antibacterial capability mainly owing to destroy the structural integrity of bacteria (Diaz-Gomez et al., 2013) or inhibit the formation of bacterial biofilm *in vitro* (Kang et al., 2008), and thus it has been widely applied in pharmaceutical and food industries (He et al., 2019a). Meanwhile, GA also shows a positive effect on antioxidant, anti-inflammatory, and anti-tumor (Verma et al., 2013; Hans et al., 2016). As Anuraga et al. (2018) reported the polyhydroxy structure of GA contributes to well protein binding ability whereby reducing proteolysis during ensiling process. Additionally, a certain amount of GA added into the ruminant diet can reduce the emission of methane and nitrous oxide in the rumen, thus improving the efficiency of ruminant feed energy utilization and also reducing greenhouse gas emissions for ecological environment protection. GA also has benefit on animals' growth performance and health in the feeding practice. Samuel et al. (2017) reported that addition of GA at 75–100 mg/kg into broiler chicks' diet improved feed conversion efficiency in both the grower and overall periods. And adding 0.1 g/L GA in drinking water can regulate the diversity of microflora of broilers, increase the abundance of *Firmicutes*, and decrease the abundance of *Bacteroidetes*, and thus promote the growth of broilers (Serajus et al., 2017). To sum up, GA might be a candidate of ideal silage additives. However, a few studies were available about the effects of GA additive on WPS silage, especially for dynamic ensiling.

Therefore, WPS was ensiled with and without 0.5 and 1% GA for a fermentation period of 3, 7, 14, and 30 days, and then fermentation quality, nitrogen fraction, and bacterial communities of the WPS silage were evaluated.

MATERIALS AND METHODS

Silage Preparation

Whole plant soybean was planted without herbicides or fertilizers and harvested with an approximately homogeneous size. During the fruiting period, WPS was hand-collected from QILIN experimental field at the South China Agricultural University (23°19′ N, 113°34′ E) in Guangzhou City, Guangdong Province, China. The samples were then immediately chopped into 2–3-cm lengths by the manual cutter (Model 9ZP-3.6, Kaiyue Machinery Company, China). After homogenization, the triplicate samples of raw material were used for determining the chemical composition and microbial populations. And the ensiling treatments were conducted with or without 1 and 2% GA (CAS: 149-91-7, purity $\geq$ 99%; Shanghai-Macklin, China mainland) on an FM basis. Following mixing WPS and GA thoroughly, about 110 g of silage materials were then packed into polyethylene bags and sealed immediately using a vacuum sealer (Lvye DZ280, Yijian Packaging Machinery Co. Ltd., China). A total of 36 silage bags (4 ensiling stage × 3 treatments × 3 replicates) were kept at room temperature (25–30°C) and randomly sampled on days 3, 7, 14, and 30 of ensiling for analysis of fermentation parameters, protein fractions, and bacterial communities.

Fermentation Characteristics and Chemical Component Analysis

The methods used in this part were similar to our previous study (Wang et al., 2020).

Hence, 20 g of individual silage sample were homogenized with distilled water (180 ml) in an orbital shaker at room temperature, and the supernatants were then serially diluted from 10^{-1} to 10^{-6}. Yeast and mold counts were incubated and counted using Rose Bengal agar at 28°C for 72–120 h. Lactic acid bacteria (LAB) and coliform bacteria were cultured respectively on de Man, Rogosa, Sharpe (MRS) agar and Violet Red Bile agar at 30°C for 48 h. Another 20 g silage sample were mixed with 180ml distilled water and incubated overnight at 4°C, then one aliquot of the filtrate was used to measure pH value with pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd., Shanghai, China).

According to Bai et al. (2020), organic acids including lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were also determined by filtrate. The ammonia-N (NH_3-N) content was detected by the phenol-hypochlorite colorimetric method (Ke et al., 2017). The surplus silages were oven-dried for calculating dry matter (DM) and ground for chemical analysis. And those chemical compounds were analyzed in triplicate and expressed on DM basis. Crude protein (CP) and true protein (TP) were analyzed by Kjeldahl nitrogen analyzer (Kjeltec 2300 Auto Analyzer, FOSS Analytical AB, Hoganas, Sweden) according to the methods of the Association of Official Analytical Chemists (AOAC, 1990). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to Van Soest et al. (1991). The content of WSCs was detected by anthrone method (Murphy, 1958). Hydrolyzable tannin (HT) was measured by the Folin–Ciocalteu colorimetric as described by Makkar (2010).

Bacterial Community Sequencing Analysis

The WPS silages were sampled and extracted the total bacterial DNA with a DNA Kit (Omega Biotek, Norcross, GA, United States) following the attached instructions. And specific conducted steps were similar to Bai et al. (2020). The V3–V4 regions of 16S rDNA were amplified using the primers (341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT). Polymerase chain reactions

(PCRs) were conducted in a 50-μl mixture [5 μl of 10 × KOD Buffer, 1.5 μl of each primer (5 μM), 1 μl of KOD polymerase, 5 μl of 2.5 mM dNTPs, and 100 ng of template DNA] and the same reaction procedures as reported by He et al. (2019a). After purified and quantified, the purified PCR products were sequenced on an Illumina HiSeq 2500 Sequencing System (Illumina, Inc., San Diego, CA, United States), and the raw sequences were analyzed according to the procedures of Wang et al. (2018). The bioinformatic data were analyzed using the free online platform[1]. The α-diversity was calculated in the Quantitative Insights Into Microbial Ecology (QIIME) (version 1.9.1) bioinformatic pipeline (version 1.8.2)[2]. The β-diversity was analyzed with principal component analysis (PCA). And the relative abundances of different bacterial communities at the phylum and genus levels were also analyzed.

Statistical Analysis

The effects of GA and ensiling days on the fermentation quality and chemical characteristics of WPS silage were analyzed with IBM SPSS 20.0 for Windows statistical software package. The results were evaluated using two-way analysis of variance (ANOVA), with Duncan's multiple range tests. Statistical significance was determined at the $P < 0.05$ level. All the figures in this paper were downloaded from Omicsmart online platform and further embellished by the software Adobe Illustrator CS 6.0.

RESULTS

Characteristics of Fresh Whole Plant Soybean Prior to Ensiling

The chemical compositions and microbial population of fresh WPS prior to ensiling were listed in **Table 1**. The DM content of WPS was 285 g/kg FM. And the nutrition indexes including CP, TP, NPN, NDF, and ADF were 172 g/kg DM, 840 g/kg TN, 160 g/kg TN, 462 and 274 g/kg DM, respectively. The WSC content of WPS was 44.7 g/kg DM. Moreover, the epiphytic LAB count of fresh WPS in this trial was 5.37 $\log_{10}$ CFU/g FM, and the counts for yeasts, molds, and coliform bacteria were 5.02, 4.49, and 5.54 $\log_{10}$ CFU/g FM, respectively. The tannin content including total phenol, SP, and HT were 3.55, 1.44, and 1.61 g/kg DM, respectively.

Fermentation Quality, Microbial Population, and Chemical Compositions of Dynamic Whole Plant Soybean Silage

The DM content, pH value, organic acids content, and microbial population of WPS dynamically ensiled with or without GA are presented in **Table 2**. The DM content was significantly improved ($P < 0.05$) with GA addition on ensiling days 3 and 30, also increased ($P > 0.05$) on days 7 and 14. The pH value of GA-treated silages was significantly ($P < 0.05$) decreased in whole ensiling stage. And ensiling days had a highly significant effect ($P < 0.01$) on pH decline. However, all treatments of WPS silage showed relatively high pH value (5.38–6.56). Meanwhile, GA addition significantly improved ($P < 0.05$) the LA and AA contents, while these contents significantly decreased ($P < 0.05$) with prolonged ensiling days (except for 1% GA treatment on AA content). The LAB count decreased significantly ($P < 0.05$) with GA addition, and the addition of GA also significantly decreased ($P < 0.05$) other microbes' number (yeasts, molds, and coliform bacteria). BA content was significantly decreased ($P < 0.05$) with GA addition but significantly increased ($P < 0.05$) with prolonged ensiling days. **Table 3** showed the protein fractions and tannin content of WPS dynamically ensiled with or without GA. In our study, the addition of GA had little effect on the content of TP and NPN. And with the prolonged ensiling days, TP content of WPS silage significantly reduced ($P < 0.05$), while NPN and NH_3-N contents significantly increased ($P < 0.05$). And the addition of GA decreased the NH_3-N ($P < 0.05$) content of WPS silage. Furthermore, the tannin content including total phenol, SP, and HT all significantly ($P < 0.05$) increased with addition of GA. But with the prolonged ensiling days, their content all significantly ($P < 0.05$) decreased.

TABLE 1 | Chemical composition and microbial population of fresh whole plant soybean prior to ensiling (±SD, $n = 3$).

Items	Whole plant soybean
Dry matter (g/kg FM)	285 ± 4.9
Crude protein (g/kg DM)	172 ± 12.8
True protein (g/kg TN)	840 ± 25.3
Non-protein nitrogen (g/kg TN)	160 ± 25.3
Neutral detergent fiber (g/kg DM)	462 ± 32.7
Acid detergent fiber (g/kg DM)	274 ± 27.2
Water soluble carbohydrate (g/kg DM)	44.7 ± 3.38
Lactic acid bacteria (Log_{10} CFU/g FM)	5.37 ± 0.12
Yeasts (Log_{10} CFU/g FM)	5.02 ± 0.06
Molds (Log_{10} CFU/g FM)	4.49 ± 0.20
Coliform bacteria (Log_{10} CFU/g FM)	5.54 ± 0.10
Total phenol (g/kg DM)	3.05 ± 4.26
Simple phenol (g/kg DM)	1.44 ± 0.28
Hydrolyzable tannin (g/kg DM)	1.61 ± 0.30

FM, fresh matter; DM, dry matter; TN, total N; CFU, colony forming units.

Bacterial Diversity of Whole Plant Soybean Silage

In this study, α-diversity of bacterial community of raw material and dynamically ensiled WPS was shown in **Table 4**. The good coverage value for all treatments were all above 0.99. And the addition of GA increased the Shannon, Simpson, Chaos, and Ace index compared to contrast check (CK). And the operational taxonomic units (OTUs), richness, and diversity of bacterial communities were significantly increased ($P < 0.05$) with prolonged ensiling days. The result of unweighted principal coordinate analysis (PCoA) was shown in **Figure 1**. PCoA 1 and PCoA 2 of WPS silage were 64.23 and 24.39%, respectively. Moreover, the bacterial community of WPS ensiled alone showed a clear separation from the samples treated with GA addition.

[1]http://www.omicshare.com/tools

[2]https://qiime.org

TABLE 2 | Organic acid contents, pH and microbial population of ensiled whole plant soybean.

Items	Treatments	Ensiling days				SEM	Significant		
		3	7	14	30		D	T	D*T
[2]Dry matter (g/kg FM)	CK	[1]279^{c}	276	276	265^{b}	1.95	NS	**	NS
	0.5%GA	291^{b}	285	294	288^{a}				
	1%GA	290^{a}	290	293	294^{a}				
pH	CK	6.56^{aB}	6.51^{aA}	6.18^{aA}	6.16^{aB}	0.06	**	**	NS
	0.5%GA	6.13^{b}	5.99^{b}	5.86^{ab}	5.74^{b}				
	1%GA	5.61^{cA}	5.58^{cA}	5.62^{cA}	5.38^{cB}				
Lactic acid (g/kg DM)	CK	18.1^{cA}	11.7^{cB}	10.4^{cC}	9.62^{bC}	4.8	**	**	**
	0.5%GA	64.3^{bA}	46.9^{bB}	24.2^{bC}	14.4^{bC}				
	1%GA	85.2^{aA}	72.1^{aA}	41.6^{aB}	31.5^{aB}				
Acetic acid (g/kg DM)	CK	54.7^{bA}	43.3^{bA}	26.9^{bB}	24.1^{cB}	4.57	**	**	**
	0.5%GA	57.8^{bB}	93.1^{aA}	91.4^{aA}	64.5^{bB}				
	1%GA	93.7^{a}	81.4^{a}	93.4^{a}	85.6^{a}				
Propionic acid (g/kg DM)	CK	ND	ND	ND	ND	—	—	—	—
	0.5%GA	ND	ND	ND	ND				
	1%GA	ND	ND	ND	ND				
Butyric acid (g/kg DM)	CK	32.0^{aC}	40.4^{aB}	52.0^{aB}	65.3^{aA}	2.96	**	**	*
	0.5%GA	8.04^{bC}	32.4^{aB}	36.4^{bB}	43.3^{bA}				
	1%GA	ND^{cD}	20.0^{bC}	29.7^{bB}	42.0^{bA}				
Lactic acid bacteria (Log10 CFU/g FM)	CK	8.72^{aA}	8.92^{aB}	8.60^{aAB}	8.36^{aB}	0.09	**	**	**
	0.5%GA	7.89^{C}	8.45^{aA}	8.53^{aA}	8.10^{bB}				
	1%GA	7.07^{cC}	7.76^{bB}	8.22^{bA}	7.79^{cB}				
Molds (Log_{10} CFU/g FM)	CK	3.93^{aA}	$<2.00^{B}$	$<2.00^{B}$	$<2.00^{B}$	0.14	**	NS	**
	0.5%GA	3.81aA	$<2.00^{B}$	$<2.00^{B}$	$<2.00^{B}$				
	1%GA	2.97^{bA}	$<2.00^{B}$	$<2.00^{B}$	$<2.00^{B}$				
Yeasts (Log_{10} CFU/g FM)	CK	3.92^{aA}	2.75^{B}	$<2.00^{C}$	$<2.00^{C}$	0.13	**	Ns	NS
	0.5%GA	3.83^{aA}	2.93^{A}	2.98^{A}	$<2.00^{B}$				
	1%GA	3.20^{bA}	2.61^{B}	2.42^{B}	$<2.00^{C}$				
Coliform bacteria (Log_{10} CFU/g FM)	CK	6.49^{aB}	7.95^{aA}	7.79^{A}	6.36^{aB}	0.17	**	**	NS
	0.5%GA	6.57^{aB}	7.62^{bA}	7.29^{A}	5.91^{aC}				
	1%GA	5.45^{bB}	7.35^{cA}	6.99^{A}	4.86^{bB}				

[1]Means in the same row ($^{A–C}$) or column ($^{a–c}$) followed by different letters differ ($P < 0.05$).
*[2]FM, fresh matter; DM, dry matter; CFU, colony forming units; ND, not detected; "—", default; SEM, standard error of means; CK, control; 0.5%GA, 0.5% FM gallic acid added; 1%GA, 1% FM gallic acid added; D, ensiling days effect; T, treatments effect; D*T, the interaction effect of treatments and ensiling days. *$P < 0.05$; **$P < 0.01$; NS, no significant effect.*

Bacterial Abundance of Whole Plant Soybean Silage

The relative abundances of bacterial communities of fresh material and WPS silages at the phylum and genus levels were presented in **Figure 2** (Circos map) and **Figure 3** (accumulation map). As seen in Circos map, *Cyanobacteria* (58.50%), *Proteobacteria* (31.44%), and *Firmicutes* (9.54%) were the top three phyla in the fresh WPS material, while *Firmicutes* (52.25%), *Proteobacteria* (31.92%), and *Cyanobacteria* (14.95%) were also top three dominant phyla in the whole WPS silages. And *Pantoea* (10.57%), *Lactobacillus* (6.46%), and *Lactococcus* (0.95%) were the most dominant genera in the fresh WPS material, but *Lactobacillus* (28.65%), *Lactococcus* (7.92%), and *Weissella* (2.88%) were the most dominant genera in all WPS silages. **Figure 3** (accumulation map) shows the abundance and variation of bacterial community in phylum and genus levels. *Cyanobacteria* was the most abundant phylum in fresh material on phylum level. The relative abundance of *Cyanobacteria* decreased, while *Firmicutes* and *Proteobacteria* increased rapidly and became the dominant phyla with prolonged ensiling days. And the relative abundance of *Cyanobacteria* and *Proteobacteria* decreased while *Firmicutes* increased with GA addition. Moreover, *Pantoea* and *Lactobacillus* were the most dominant genera in fresh WPS. However, the relative abundance of *Pantoea* decreased (below 2% in all treatments) during the ensiling process. The most dominant genus in WPS silage was *Lactobacillus*, followed by *Lactococcus*, *Weissella*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. And the GA additive increased the abundance of *Lactobacillus*, while it decreased the abundance of *Lactococcus* and *Weissella*.

DISCUSSION

Characteristics of Fresh Whole Plant Soybean Prior to Ensiling

The DM content of WPS was approximate to ideal DM (30–35%) for satisfactory silage (Guyader et al., 2018). As McDonald et al. (1991) and Muck (2010) reported, high moisture content of fresh material is considered to increase fermentation of undesirable microorganisms, mainly for *Clostridium*, which would result in the nutrition loss of effluent and spoilage during the ensiling

TABLE 3 | Protein fractions and tannin content of ensiled whole plant soybean.

Items	Treatments	Ensiling days				SEM	Significant		
		3	7	14	30		*D*	*T*	D*T
[2]Crude protein (g/kg DM)	CK	167	168	174	169	1.28	NS	NS	NS
	0.5%GA	163	169	178	176				
	1%GA	163	170	162	172				
True protein (g/kg TN)	CK	[1]567^{A}	488^{B}	458^{C}	384^{D}	12.1	**	NS	NS
	0.5%GA	554^{A}	486^{B}	465^{B}	385^{C}				
	1%GA	568^{A}	550^{A}	445^{B}	386^{B}				
Non-protein-N (g/kg TN)	CK	433^{D}	512^{C}	542^{B}	616^{A}	12.1	**	NS	NS
	0.5%GA	446^{C}	514^{B}	535^{B}	615^{A}				
	1%GA	432^{B}	450^{B}	555^{A}	614^{A}				
Ammonia-N (g/kg TN)	CK	38.1^{aC}	87.9^{aC}	125^{aB}	259^{aA}	11.6	**	**	**
	0.5%GA	16.0^{bC}	38.2^{bC}	67.5^{bB}	112^{bA}				
	1%GA	10.4^{bC}	22.9^{bC}	50.8^{bB}	88.2^{bA}				
Total phenol (g/kg DM)	CK	6.05^{bA}	4.98^{bB}	3.65^{cB}	2.50^{bC}	0.83	**	**	NS
	0.5%GA	14.0^{aA}	6.88^{bB}	9.20^{bB}	5.75^{abB}				
	1%GA	18.0^{aA}	12.7^{aB}	11.5^{aB}	10.2^{aB}				
Simple phenol (g/kg DM)	CK	4.17^{aA}	3.23^{AB}	2.22^{B}	2.08^{cB}	0.31	**	**	**
	0.5%GA	5.11^{aA}	3.21^{B}	3.14^{B}	3.57^{bB}				
	1%GA	8.27^{bA}	2.75^{B}	4.08^{B}	6.77^{aA}				
Hydrolyzable tannin (g/kg DM)	CK	1.88^{a}	1.75b	1.43b	0.43	0.64	**	**	NS
	0.5%GA	8.87^{bA}	3.67^{bB}	6.07^{aAB}	2.19^{B}				
	1%GA	9.71^{bA}	9.93^{aA}	7.40^{aAB}	3.44^{B}				

*[1]Means in the same row ($^{A-D}$) or column ($^{a-c}$) followed by different letters differ ($P < 0.05$). [2]DM, dry matter; TN, total N; SEM, standard error of means; CK, control; 0.5%GA, 0.5% FM gallic acid added; 1%GA, 1% FM gallic acid added; D, ensiling days effect; T, treatments effect; D*T, the interaction effect of treatments and ensiling days. **$P < 0.01$. NS, no significant effect.*

TABLE 4 | Alpha diversity of bacterial community of ensiled whole plant soybean.

Items	[2]FM	Treatments	Ensiling days				SEM	Significant		
			3	7	14	30		D	T	D*T
Sobs	884	CK	[1]984^{a}	938	927	1020^{b}	25.3	**	*	**
		0.5%GA	900^{bB}	977^{B}	914^{B}	1437^{aA}				
		1%GA	968a	954	952	1178^{b}				
Shannon	3.21	CK	4.83	4.80	4.89	5.39^{b}	0.13	**	NS	NS
		0.5%GA	3.99^{C}	5.28^{B}	5.17^{B}	6.07^{aA}				
		1%GA	4.66^{B}	5.36^{AB}	5.40^{AB}	6.00^{aA}				
Simpson	0.66	CK	0.89	0.89	0.87	0.92	0.01	**	*	**
		0.5%GA	0.76^{B}	0.93^{A}	0.93^{A}	0.95^{A}				
		1%GA	0.88^{B}	0.94^{A}	0.94^{A}	0.96^{A}				
Chao	1409	CK	1572	1528	1527C	1627^{b}	28.8	**	*	*
		0.5%GA	1536	1747	1462	2016^{aA}				
		1%GA	1658	1595	1597	1753^{b}				
Ace	1415	CK	1593	1553	1598	1684^{b}	33.2	**	*	*
		0.5%GA	1579^{B}	1756^{B}	1525^{B}	2140^{aA}				
		1%GA	1720	1619	1637	1898^{ab}				
Goods-coverage	1.00	CK	0.99	1.00	1.00	0.99	—	NS	NS	NS
		0.5%GA	1.00	0.99	0.99	0.99				
		1%GA	0.99	0.99	0.99	0.99				

*[1]Means in the same row ($^{A-C}$) or column ($^{a-c}$) followed by different letters differ ($P < 0.05$). [2]FM, fresh soybean straw; CK, control; 0.5%GA, 0.5% FM gallic acid added; 1%GA, 1% FM gallic acid added; SEM, standard error of means; "—", default; D, ensiling days effect; T, treatments effect; D*T, the interaction effect of treatments and ensiling days. **$P < 0.01$. NS, no significant effect.*

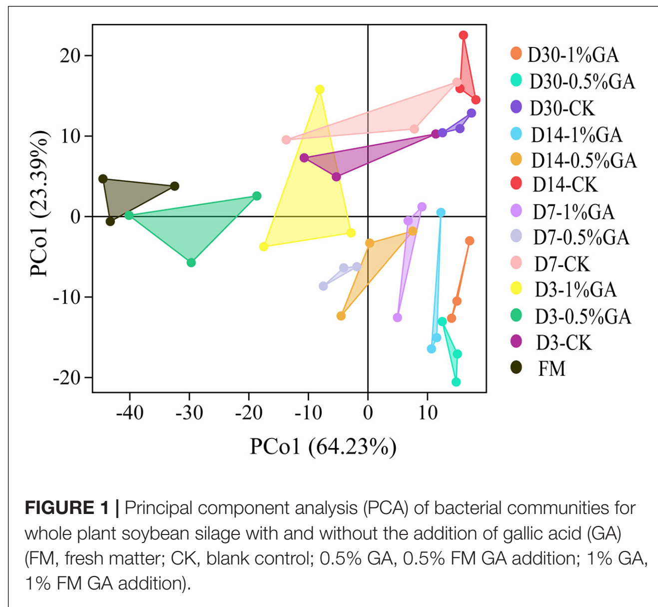

FIGURE 1 | Principal component analysis (PCA) of bacterial communities for whole plant soybean silage with and without the addition of gallic acid (GA) (FM, fresh matter; CK, blank control; 0.5% GA, 0.5% FM GA addition; 1% GA, 1% FM GA addition).

process. In general, the WPS is nutrition abundant with high protein content and appropriate fiber content. Especially for high proportion of TP, it means that more valuable protein fraction will be higher efficient in utilization for livestock compared to NPN (He et al., 2019b). However, the protein and fiber contents of WPS were discrepant with those shown by Khorvash et al. (2010). Such variations of the chemical composition of the forage might contribute to the plant varieties, geographical location, climate, fertilization, and season of harvest (Zhang et al., 2016).

The sufficient WSC content and epiphytic microbial community (uppermost for LAB) of raw material are the two conclusive factors for well silage quality. The WSC is the fermentation substrate, which is mainly utilized by bacteria to produce organic acid and thus decrease silage pH value. In our study, the WSC content of WPS was not abundant and less than 60–70 g/kg DM, the theoretical requirement for obtaining well-preserved silage (Smith, 1962). Moreover, the epiphytic LAB count of fresh WPS was comparative to 5 $\log_{10}$ CFU/g FM, which is necessary to produce well-quality silage (Cai et al., 1998). However, the counts for undesirable fungi and coliform bacteria were relatively high, which indicated that LAB might be difficult to occupy a dominant position at the early stage of ensiling. Furthermore, the HT in WPS might be helpful for undesirable microorganism inhibition (Peng et al., 2017) and extensive proteolysis limitation (Anuraga et al., 2018). To sum up, the WPS was hard to ensile directly because of the insufficient WSC content and high undesirable microbial communities if no silage additives.

Fermentation Quality, Microbial Population, and Chemical Compositions of Dynamic Whole Plant Soybean Silage

The DM content was improved with GA addition. It indicates GA had a positive effect on DM preservation. As Ávila et al. (2014) reported, the silage DM was mainly consumed by respiration of plant cell and aerobic activity, such as the metabolism of *Clostridia* or yeasts at the early stage of ensiling. He et al. (2019a) also reported dry matter loss of stylo reduced with GA addition after 30 days' ensiling. In this study, all treatments of WPS silage showed relatively high pH value, which were

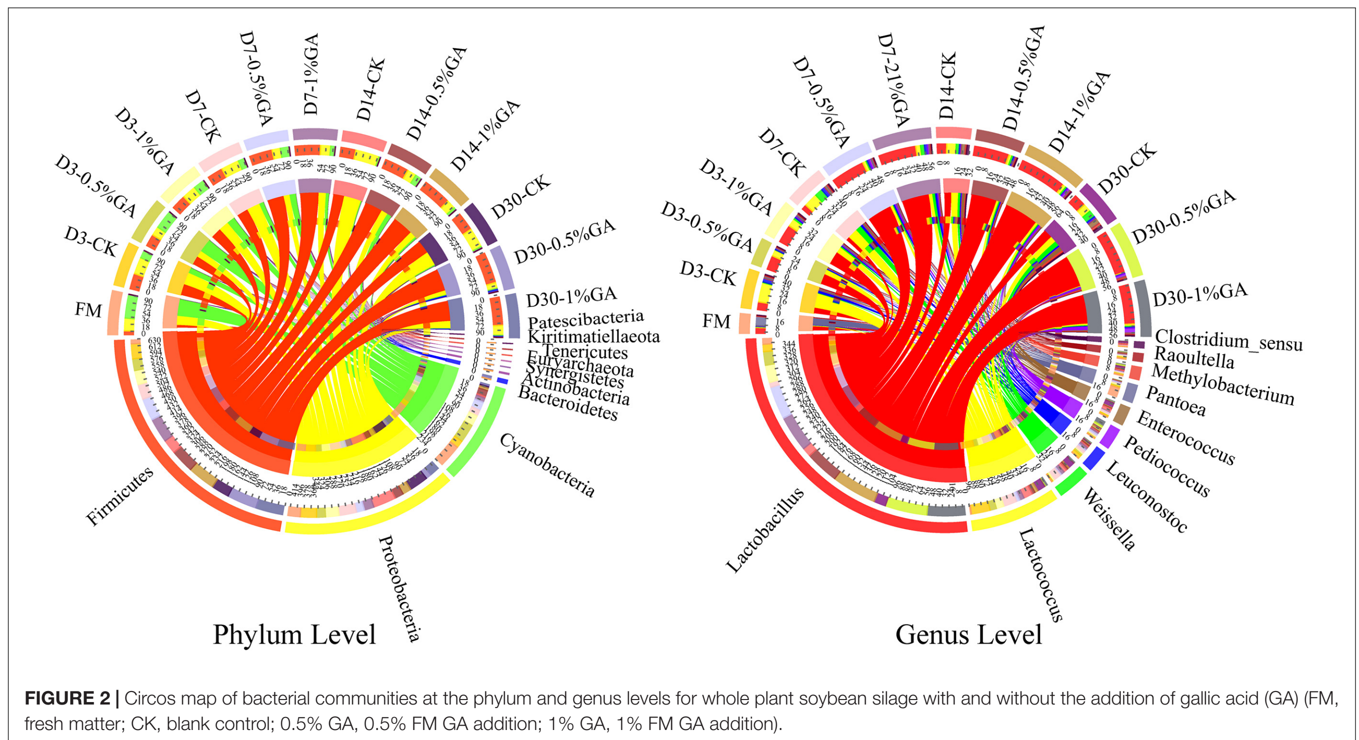

FIGURE 2 | Circos map of bacterial communities at the phylum and genus levels for whole plant soybean silage with and without the addition of gallic acid (GA) (FM, fresh matter; CK, blank control; 0.5% GA, 0.5% FM GA addition; 1% GA, 1% FM GA addition).

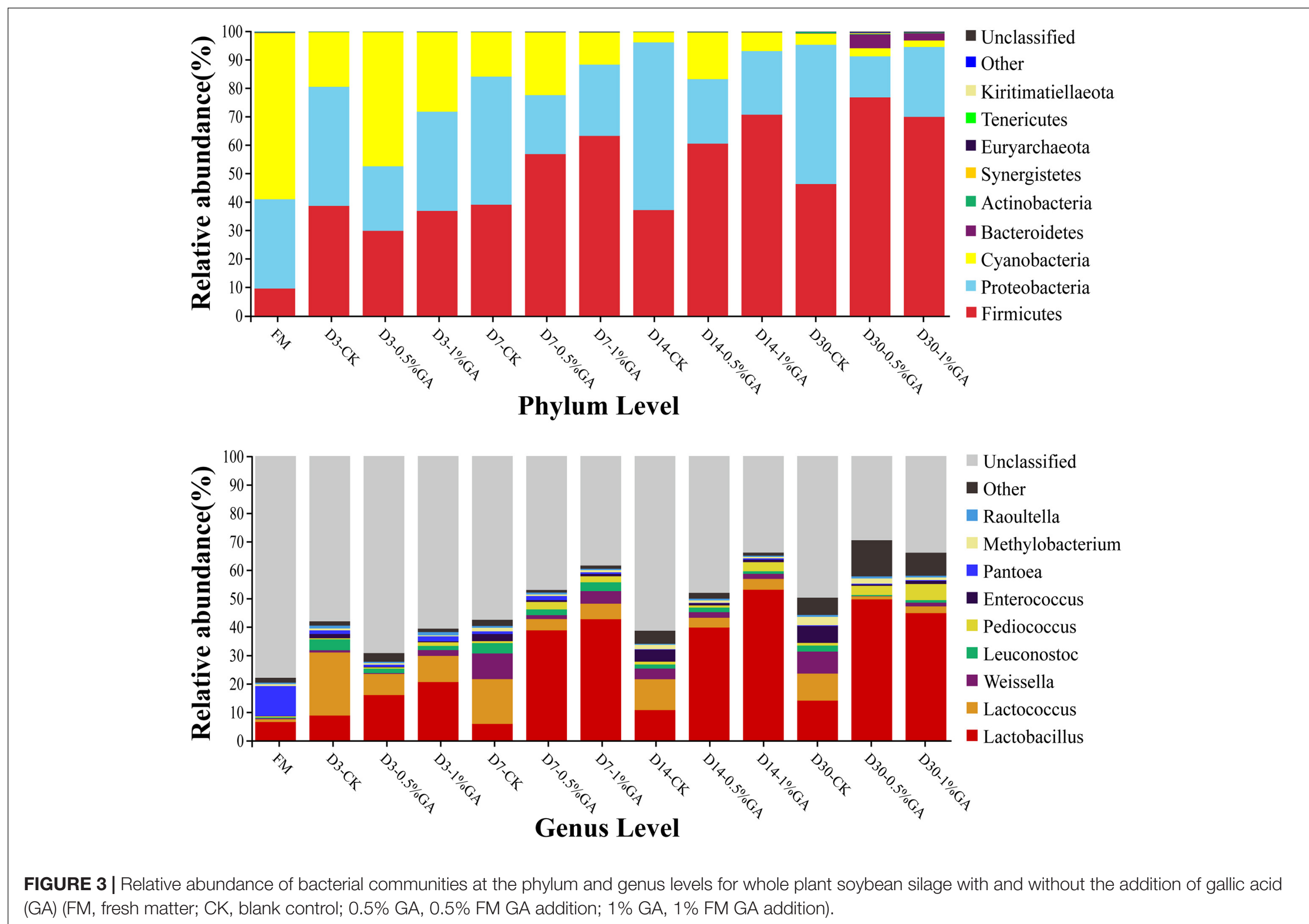

FIGURE 3 | Relative abundance of bacterial communities at the phylum and genus levels for whole plant soybean silage with and without the addition of gallic acid (GA) (FM, fresh matter; CK, blank control; 0.5% GA, 0.5% FM GA addition; 1% GA, 1% FM GA addition).

much higher than 4.2, an indicator of well-preserved silage and thus not suitable for aerobic stability and biomass preservation. Ensiling days decreased the pH value; it was ascribed to the accumulation of organic acid. As well, the pH value of GA-treated silages was decreased throughout the whole ensiling stage. The pH value of silage generally decreases with accumulating of organic acids, which are converted by various microorganisms to ferment WSC. Kung et al. (2018) reported the pH of silages is greatly influenced by acid concentration and the buffering capacity of materials. Therefore, the relative high pH value of WPS silage in our study might be because of the presence alkaline substances like ammonia-N generated from proteolysis or the buffering capacity blocked its decline (He et al., 2019a). Meanwhile, GA addition improved the LA and AA contents. LA is the production of LAB fermentation and charge of pH decline during the early stage of ensiling, while the AA is mainly caused by hetero-fermentative LAB (McDonald et al., 1991). It might be owed to the antibacterial property of GA, which inhibits the undesirable bacteria and benefits LAB fermentation and then reduces negative nutrient competition. Moreover, the content of AA was much higher than LA, especially in the later stage of ensiling. Parvin and Nishino (2009) reported that the growth of AA-producing bacteria might metabolize LA to AA under sugar-deficient conditions. And the AA was an inhibitor of the growth of spoilage microorganisms and increased the aerobic stability (Seglar, 2003). The microbial population presented in **Table 2** can further prove the antibacterial effect of GA. The LAB was the dominant microbe in all silages, and its count decreased significantly ($P < 0.05$) with GA addition. This might be caused by decreasing of cocci (such as *Leuconostocs*, *Pediococcus*, *Lactococci*, and *Enterococci*), which was less tolerant to low pH (Cai et al., 1998; Pang et al., 2011). And the addition of GA also decreased yeasts, molds, and coliform bacteria count. By contrast, BA content was decreased with GA addition but increased with prolonged ensiling days. In addition, PA was not detected in all silages. BA is undesirable in silage because of the nutritional damage caused by secondary fermentation as a result of *clostridial* activity (McDonald et al., 1991). In this study, the high BA content might be owed to the abundant coliform bacterial count (4.86–7.95 Log_{10} CFU/g FM), which cannot be restricted in such a high pH condition. According to the above analysis, GA addition is helpful to improve fermentation quality of WPS silage.

The primary purpose of silage-making is to conserve biomass and reduce nutrient loss of fresh forage (Wang et al., 2020). The CP content of WPS silages was comparable to mulberry leaf silage (Wang et al., 2019) but much higher than stylo silage (He et al., 2019a). It indicated that WPS can be utilized as a desirable silage material for the purpose of reducing the

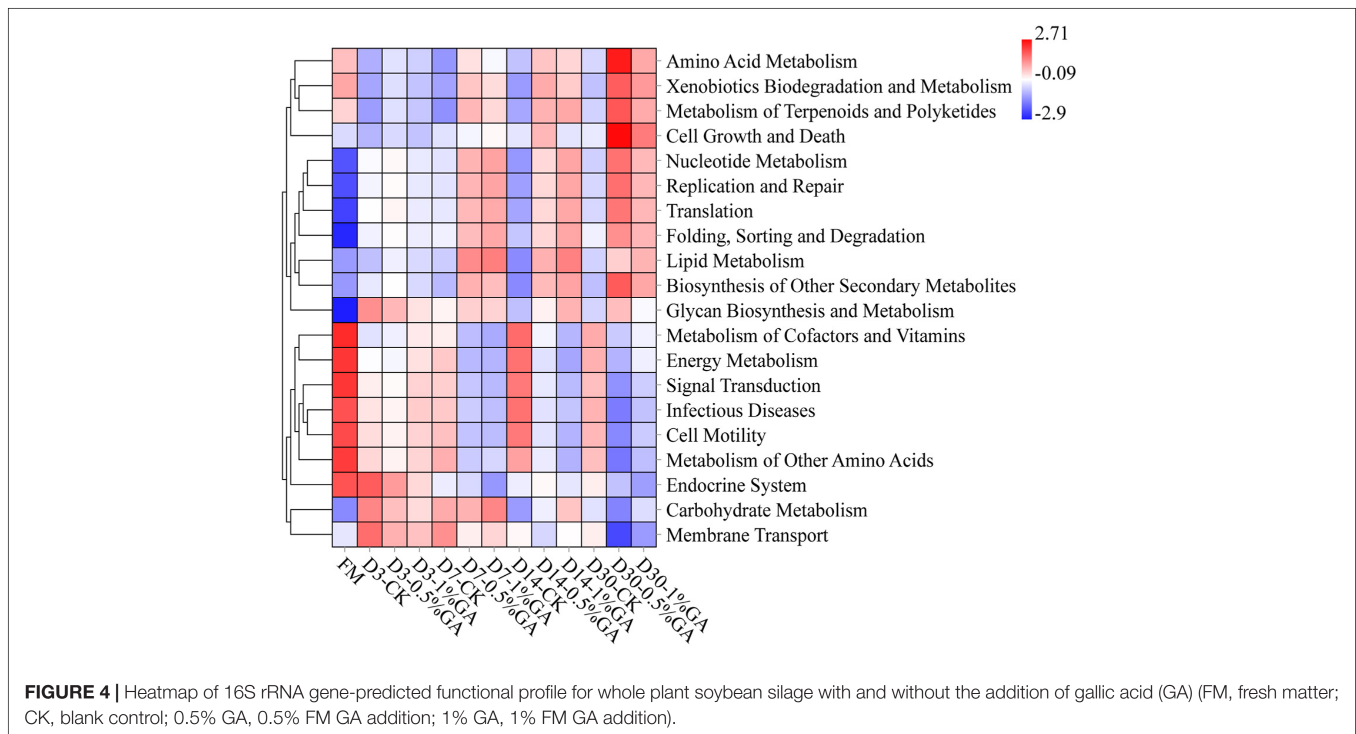

FIGURE 4 | Heatmap of 16S rRNA gene-predicted functional profile for whole plant soybean silage with and without the addition of gallic acid (GA) (FM, fresh matter; CK, blank control; 0.5% GA, 0.5% FM GA addition; 1% GA, 1% FM GA addition).

waste of biomass resources. In principle, ensiling is a dynamic enzymatic and microbial reaction process, and one of the most important is proteolysis. Proteolysis also called protein degradation, which transforms TP to NPN, mainly some small peptides, amino acid free nitrogen, and NH_3-N (He et al., 2019a). As a result, proteolysis reduces the silage nutrition and produces terrible smell, especially for high-protein legume. Moreover, excessive rumen-degradable protein cannot be utilized by rumen microorganisms and then discharged by animal excreta (Wang et al., 2019). In the present study, more than half of the protein (840–384 g/kg DM of TP) was degraded before and after ensiling, suggesting that effective strategies should be applied to decelerate or prevent such proteolysis during the ensiling process for the purpose of preserving silage nutrition and protecting the environment by minimizing N emissions (Anuraga et al., 2018). In our study, the addition of GA had litter effect on the content of TP and NPN. The similar results also be reported by He et al. (2019a), the addition of GA decreased TP content while increased NPN content in mulberry leaves and stylo silages. And with the prolonged ensiling days, TP content of WPS silage reduced, while NPN and NH_3-N content increased. The study of Tao et al. (2012) also reported that proteolysis was the sustained response on the whole ensiling stage. Meanwhile, dipeptidase, carboxypeptidase, and tripeptidyl-peptidase were the principal exopeptidases responsible for silage proteolysis, and their optimal pH values were 8.8, 5.0, and 7.0, respectively. Therefore, the relative high pH value of WPS silage might be responsible for the continuous proteolysis. The NH_3-N content in silage is a crucial indicator of protein breakdown (Pahlow et al., 2003), which can reflect the silage quality. The addition of GA decreased the NH_3-N content, it might be due to the potential protein-binding capability of GA. Additional, Mueller (2006) reported tannins had an extensive range of antimicrobial activity. Therefore, GA additive might cause the restriction of deamination of peptides or amino acids in WPS silage and then reduced the generation of NH_3-N. To sum up, the low proportions of NH_3-N in the GA-treated group indicate that peptides and free amino acid might account for the abundant NPN. Many studies have proved the bioactivity of peptides and free amino acid on livestock growth and health (Brogden et al., 2003). Thus, the nutrition value of WPS silage was well preserved with GA addition. Surely, the tannin content increased with addition of GA. But with the prolonged ensiling days, their content all decreased. It was the same with He et al. (2019a), who reported that the HT was mostly degraded during mulberry leaf and stylo ensiling. Moreover, the residual GA of WPS silage could be further used to change the N excretion type of cattle and reduce the emission of environmental pollutants, NH_3 and N_2O (Getachew et al., 2009). Thus, the positive effect on NH_3-N restriction indicated that GA additive was effective in preserving protein of WPS silage.

Bacterial Diversity of Whole Plant Soybean Silage

In order to detect the microbial community's compositions and abundance (Ni et al., 2019) or monitor the change of microbial community in a dynamically ensiling period (Yang et al., 2018), next-generation sequencing has been widely applied in forage silage. First of all, the good coverage values for all treatments were all above 0.99, indicating that the data from sampling were adequate to represent all of the bacterial communities in the different samples. The bacterial α-diversity of each treatment was estimated by OTUs (Sobs), richness (Chao1 and Ace indexes),

and diversity (Shannon and Simpson indexes). In general, the OTUs, richness, and diversity of WPS silage were all higher than fresh WPS. Similar result was also reported by Wang et al. (2019)—higher OTUs, Chaos and Shannon index were exhibited in mulberry leaf silage relative fresh material. It indicated silage fermentation is a dynamic microbe reaction and microbial community structure, diversity, and function will be much variational before and after ensiling (Sepehri and Sarrafzadeh, 2019). Furthermore, the addition of GA increased the Shannon, Simpson, Chaos, and Ace indexes compared to CK. Many studies declared that GA had antibacterial function, while bacterial biodiversity had no change in this study. The reason for this result might be relative high pH value in all silages (>5.32) cannot inhibit the growth of poor adaptability to the acid condition bacteria, such as *Clostridium* (Ni et al., 2017). And the OTUs, richness, and diversity of bacterial community were further increased with prolonged ensiling days. But opposite results have been found by He et al. (2019b), who ensiled *Neolamarckia cadamba* leaves with formic acid and *Lactobacillus farciminis* and indexes of bacterial community (Chaos, Ace, Simpson, and Shannon) in all silages decreased with prolonged ensiling days from 3 to 60. This might ascribe to the relatively low pH value of *Neolamarckia cadamba* leaves silage (<4.31 in all silages), and it further decreased from ensiling days 3 to 60. Therefore, in this study, the GA and ensiling days had no inhibition effect on α-diversity of bacterial community mainly owing to the high pH value of the WPS silage.

The result of unweighted PCoA could reflect the distinction of the bacterial community of each treatment. Especially, a clear separation between GA-treated group and CK group, it suggested that GA had a significant influence on bacterial community. Moreover, a higher extent of separation was formed between fresh material and WPS silages. He et al. (2019a) also reported that GA had an impact on microbial community. Thus, the variations of microbial communities were strongly associated with different silage qualities (Wang et al., 2020). Surely, WPS ensiled with 0.5 and 1% GA showed a better fermentation quality that might be due to the GA-treated group promoting the microbial communities' composition and abundance and then achieving a better bacterial diversity for desirable silages.

Bacterial Abundance of Whole Plant Soybean Silage

In the present study, the variational composition of dominant phylum and genus between fresh WPS material and WPS silage in the whole ensiling period indicated that the composition and abundance of bacterial community were much altered before and after ensiling. Same results were also found by Wang et al. (2019), who reported the change of bacterial abundance between raw material and mulberry leaves silage. Accordingly, we have reason to suspect that it might be these specific variations of bacterial community that result in the change of raw material's chemical composition after the ensiling process.

Therefore, the accumulation map could clearly show the abundance and variation of bacterial community in phylum and genus levels with different treatments. Overall, on the phylum level, *Cyanobacteria* was the most abundant phylum in fresh material. Zhang et al. (2019) also reported similar results. With prolonged ensiling days, the relative abundance of *Cyanobacteria* decreased, while *Firmicutes* and *Proteobacteria* increased rapidly and became the dominant phyla; these even constituted more than 90% of relative abundance on ensiling day 30. Liu et al. (2019) also found that *Firmicutes* and *Proteobacteria* were the most dominant phyla and occupied up to 99% of the total relative abundance at the later period of barley silages. Moreover, the relative abundance of phyla between GA-treated group and CK group was much different and which was dramatically altered by the ensiling days. The relative abundance of *Cyanobacteria* and *Proteobacteria* decreased, while *Firmicutes* increased with GA addition. It was indicated that the bacterial community of WPS silage was remarkably altered by the addition of GA. The study of Heberline (2017) declared that *Cyanobacteria*, a photosynthesizing phylum of bacteria could exist in diverse environments and mainly be researched on the biotechnical and pharmaceutical industries, such as biofuels or fertilizer generation. In this study, with the effect of GA addition and ensiling days, the dominance of *Firmicutes and Proteobacteria* on the phylum level might be because of the relative low pH of WPS silages and anaerobic conditions, which were beneficial to the growth of *Firmicutes* and *Proteobacteria*. The genus-level bacterial communities of WPS silages are also shown in **Figure 3**. In general, over 75% bacterial community in fresh material was unclassified, whereas more bacterial communities (30.68–70.39%) were identified in WPS silages. It further proved the variation of fermentation parameters was mainly owed to the discrepancy of bacterial communities between fresh material and WPS silages. Moreover, *Pantoea* and *Lactobacillus* were the most dominant genera in fresh WPS. However, the relative abundance of *Pantoea* decreased during the ensiling process. Ogunade et al. (2018) found that *Pantoea* had the ability to reduce NH_3-N content and pH value in alfalfa silage. But the specific function of *Pantoea* needs to be extensively investigated in various types of forage silage. Furthermore, the most dominant genus in WPS silage was *Lactobacillus*, followed by *Lactococcus*, *Weissella*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. And the GA additive increased the abundance of *Lactobacillus*, while it decreased the abundance of *Lactococcus* and *Weissella*. Generally, *Lactobacillus*, *Weissella*, *Enterococcus*, and *Lactococcus* are the most commonly used as lactate-producing bacteria in silage and mainly used to occupy the dominant microbe position at the early stage of ensiling and ensure well fermentation quality (Pahlow et al., 2003; Yang et al., 2016; Kuikui et al., 2018). *Lactobacillus* is a rigorous homofermentative LAB and can produce two molecules of LA by decomposing one molecule of glucose. *Lactobacillus* grows rapidly and decreases pH of silage after some plant cell and aerobic microorganisms consume oxygen at the early stage of ensiling; finally, undesirable microorganisms like *Clostridium* are inhibited (Dunière et al., 2013). *Weissella* is thought to be early colonizer microorganisms and be inhibited ultimately by the low pH value (<4.20) due to organic acid accumulation (Graf et al., 2016). Because of the relatively high pH value (>5.38 in all samples) of WPS silage, *Weissella* species was detected in two experiments. Different from us, Wang et al. (2019) detected

the bacterial communities of corn stalk silage with the terminal pH of 3.86–3.98 and *Weissella* was not found. Meanwhile, most *Weissella* species are obligate heterofermentative bacteria that mainly convert WSC to lactate and acetate. Thus, the pretty high AA content (>24.1 g/kg DM) in all WPS silages might be due to the abundant *Weissella*. *Enterococcus* is also applied in grass silage as LAB inoculation, it could reduce ammonia N content of silage and directly affect ruminal fermentation by improving ruminal microbial biomass production (Weinberg et al., 2003). The high relative abundance of those genera indicated that acid production accumulated and pH reduced rapidly; therefore, the undesirable microorganisms like *Clostridium* were inhibited and the forage nutrition would be better preserved. Interestingly, *Clostridium* was not detected in our study. *Clostridium* is an undesirable genus during the ensiling process, and many studies have found that it is harmful to forage protein conservation and DM protection and produces satisfactory organic acid and thus prevents rapid fall of silage pH, whereas it promotes the growth of less acid-tolerant spoilage microorganisms (Zheng et al., 2017; He et al., 2019a). Moreover, *Clostridium* is sensitive to pH value. It can grow in conditions of pH over 4.5 and be rapidly inhibited if the pH value falls to 4 or below (Muck, 2010). The similar result found by Wang et al. (2019), *Clostridium* was also not detected in mulberry leaves silage with or without *Lactobacillus casei* and sucrose addition after 30 days' ensiling. It might be the *Clostridium* in fresh WPS, which was either found in the present study. Surely, in order to explain the occasionally unfound *Clostridium* in silages, a more advanced sequencing technique is required to classify the microbial community at a higher taxonomic level, as 16S DNA full-length sequencing or metagenomic sequencing. In addition, considerable abundance of *Leuconostoc*, *Pediococcus*, and *Methylobacterium* was found in control silage or in the GA-treated silage. Pahlow et al. (2003) reported *Leuconostoc* is also lactate-producing bacteria same as *Lactobacillus* and commonly found in silages. Therefore, the high relative abundance of *Leuconostoc* in groups was beneficial for organic acid accumulation and pH decline and then contributes to more acid-tolerant LAB (mainly for *Lactobacillus*). *Pediococcus* is often found living in association with plant material, dairy products, and foods produced by LAB (Gashe, 1985). And Cai et al. (1998) found that *Pediococcus* was suitable as a potential silage inoculant and more effective than *Lactobacillus casei.* In our study, *Pediococcus* was more abundant in the GA-treated group. The WPS silage with GA addition showed better fermentation quality, which might be explained by the abundance of desirable genera, such as *Pediococcus* and *Lactobacillus*. *Methylobacterium* is strictly neutrophilic and an aerobic bacterium and has also been found in many forage silages (Ogunade et al., 2018; Wang et al., 2019). Holland (1997) declared that *Methylobacterium* is crucial in environmental carbon cycle and contributes to the ability to metabolize plant decomposition compounds. However, the specific function of *Methylobacterium* on silages needs further study.

The 16S rRNA gene-predicted functional heatmap obtained with Tax4Fun on classification level 2 was shown in **Figure 4**. The discrepancy of bacterial composition and abundance in respective treatment groups might be the key reason for differences of gene-predicted functions. Overall, during ensiling days 7–30, WPS ensiled with the GA addition, the function of amino acid metabolism, xenobiotics biodegradation and metabolism, metabolism of terpenoids and polyketides, nucleotide metabolism, replication and repair translation folding, sorting and degradation, lipid metabolism, biosynthesis of other secondary metabolites all increased, whereas the function of energy metabolism, signal transduction, infectious diseases, cell motility, and metabolism of other amino acids all decreased. It might be the GA additive, which induced the abundant variation of some functional bacteria. For instance, the functions of metabolism of terpenoids and polyketides and the biosynthesis of other secondary metabolites increased; it might be because of the bioactivity of GA. Moreover, the antibacterial characteristic of GA could explain the function of decreased infectious diseases. Above all, GA could be applied in WPS silage to inhibit infectious diseases and improve the biosynthesis of other secondary metabolites for well fermentation quality. But further research is needed to define the other gene-predicted functions.

CONCLUSION

This present study showed that pH value, coliform bacteria count, and BA and NH_3-N contents were decreased, while LA, AA, and tannin (total phenol and HT) contents were increased with addition of GA. Meanwhile, with prolonged ensiling days, the pH value, microbe number, and LA, AA, TP, and tannin contents all decreased, whereas BA, NPN, and NH_3-N contents increased. The GA also contributes to the change of bacterial diversity, where *Firmicutes* and *Lactobacillus* were most abundant on phylum and genus levels, respectively. The above results suggested that GA additive applied in WPS silage was an effective strategy to protect forage nutrition and improve silage quality, and the 1% GA addition showed a better effect.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CW contributed to the investigation, software, data curation, formal analysis, and writing the original draft. MZ and XZ contributed to the investigation, methodology, visualization, and validation. SW contributed to the investigation and methodology. XZ contributed to the investigation, methodology, visualization, and validation. XC contributed to the conceptualization, funding acquisition, project administration, resources and validation. LG contributed to the materials, investigation, funding acquisition and methodology. QZ contributed to the conceptualization,

data curation, project administration, supervision, and validation. All authors contributed to the article and approved the submitted version.

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Melatonin Is a Promising Silage Additive: Evidence From Microbiota and Metabolites

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The safe and effective storage of forage are very important. As an important storage method, ensiling can keep fresh forage for a long time with less nutritional loss. Melatonin has antioxidant and bacteriostasis, usually used as a natural preservative. The influence of melatonin on silage microbial or fermentation quality has not been clarified. In the present study, we aimed to clarify whether melatonin affected stylo (*Stylosanthes guianensis*) silage quality via microbiota and metabolites. Melatonin addition significantly improved the silage fermentation quality, including the increased contents of lactic acid and total acid (244.18–255.81% and 63.95–78.97%, respectively), as well as the decreased in pH and butyric acid content compare with control group. Moreover, 16S rRNA sequencing indicated that melatonin addition enhanced the silage microbial diversity indices (such as increase in Shannon indices but decrease in Simpson indices), and significantly shaped the composition of silage microbiota (such as increased abundances of *Pantoea*, *Stenotrophomonas*, *Sphingobacterium,* and *Pseudomonas*, and decreased abundance of *Weissella*). Melatonin addition also dramatically affected the metabolites of sylo silage, such as raised malonic acid and some amino acid metabolism(glycine, threonine, methionine and ornithine), while reduced nucleic acid metabolism(2-deoxyuridine and thymine) and carbon metabolism(allose and 2-deoxy-D-glucose). Collectively, our results confirmed that the lowest melatonin addition (5 mg/kg) could improve the fermentation quality, and the potential mechanisms might be associated with the microbiota and metabolites in stylo.

Keywords: melatonin, stylo silage, metabolomics, bacterial community, fermentation quality

INTRODUCTION

As a naturally occurring indoleamine, melatonin is widely distributed in animals and plants, and it has a critical function in regulating their growth and development (Reiter et al., 2015). Melatonin has been studied deeply in animals, which is reported to be involved in a variety of physiological regulation processes, such as circadian rhythm, photoperiodic response, anti-aging response, immune function and oxidative stress (Tan et al., 2003, 2012). It is often used to improve sleep quality and employed as antioxidant to treat neurasthenia (Reiter et al., 2015, 2016). Recently, the biological function of melatonin in plants has also attracted wide attention.

Melatonin is considered as an active oxygen scavenger, which can effectively scavenge reactive oxygen radicals, inhibit peroxidation, delay plant senescence, and alleviate salt, drought, heavy metal, cold, pathogens and other stresses (Kang et al., 2010; Yin et al., 2013; Zhang et al., 2013; Bajwa et al., 2014; Lee et al., 2014; Wang et al., 2014; Wei et al., 2015, 2017, 2020).

In recent years, more and more attention has been paid to food safety. Therefore, how to store crop products safely and effectively has become a hot research topic. Melatonin, as a natural preservative, has freshness-retaining effect, and it is widely used in the storage of fruits, vegetables and other crops. Melatonin can promote the ripening and quality of tomato fruit (Sun et al., 2014), delay the postharvest senescence, improve the cold resistance of peach fruit (Cao et al., 2016; Gao et al., 2017), reduce the postharvest decay of strawberry fruit and retain its nutritional value (Aghdam et al., 2019), and reduce the physiological degradation of cassava root after harvest (Ma et al., 2016).

Animal products are an important source of food, and feed safety is closely related to the quality of animal products. Under the background of global climate change, how to keep green forage safely and efficiently has become a great challenge for animal husbandry all over the world. Ensiling is an approach for long-term preservation of green forage under the anaerobic conditions, which is an ancient agricultural production approach used more than 3,000 years (Wilkinson et al., 2003; Kung et al., 2018). Generally, the characteristics of desirable silage were lower pH value and higher lactic acid content. Because both pH and organic acids (especially lactic acid) were important indexes to evaluate silage fermentation quality. In particular, the desirable legumes forage silage(Dry matter was <30–35%), the pH should be below 4.3–4.5, and the organic acids were mainly lactic acid (6–8%), that means forage well preservation(Kung et al., 2018).

Additives have been widely used to enhance the preservation of silage. Common additives include lactic acid bacteria (LAB) or other inoculants, chemicals, and enzymes (Muck et al., 2018). Nevertheless, the activities of biological additives have higher requirements on the environment, and the chemical additives will cause certain environmental problems and animal health concerns (Muck et al., 2018). Melatonin, as a natural preservative, has reliable safety in fruit and food storage, and it also has great potential for silage additives (Sun et al., 2014; Cao et al., 2016; Ma et al., 2016; Gao et al., 2017; Aghdam et al., 2019). However, the effect and mechanism of melatonin on silage fermentation quality are still unclear.

Stylo (*Stylosanthes guianensis*) is a very important legume forage widely distributed in the tropical and subtropical regions of the world, which is an important feed source for local livestock (Li et al., 2017). Due to the influence of climate and environmental factors, stylo production is highly seasonal, which limits its popularization and utilization and ensiling is really essential to ensure the balanced annual supply of livestock feed. Conventional additives have been reported in stylo silage, which can improve the fermentation quality and affect the microbial community of silage (Li et al., 2017; He et al., 2020c). In view of the characteristics of melatonin and its application in fruit and food preservation, we hypothesized that melatonin could regulate the fermentation quality of stylo silage by altering the microorganisms and metabolites. However, the effect of melatonin on silage fermentation of stylo is unknown. Herein, in this study, we detected the fermentation quality, microbial community and metabolites of stylo silage treated with different level of melatonin, in order to explore the effect of melatonin on silage and its possible mechanism.

MATERIALS AND METHODS

Silage Processing

Stylo was cultivated at our experimental base (109°58'E, 19°52'N, Chinese Academy of Tropical Agricultural Sciences). The vegetative period stylo was harvested and cut into tiny sections (about 2 cm). Melatonin (BC Grade, Purity ≥ 99.0%) was obtained from Sangon Biotech Co., Ltd. (Shangshai, China). Four different treatments were conducted in our current study as follows: (Reiter et al., 2015) no additive (CK), (Tan et al., 2003) 5 mg/kg melatonin (Mela1) (Tan et al., 2012), 10 mg/kg melatonin (Mela2), and (Reiter et al., 2016) 20 mg/kg melatonin (Mela3). The application rate of melatonin was calculated based on fresh matter. Every treatment was carried out in triplicate. Briefly, 500 g of stylo and melatonin (powder) was blended and shook well, and the mixture was placed and vacuumed in plastic bags (50 cm × 20 cm × 10 cm; Guozhong Packing Co., Ltd., Haikou, China). A total of 12 bags (four treatments × three replicates) were prepared and incubated at normal temperature (25–30°C). The organic acid, microbial community and metabolites were determined after 30 days of fermentation.

Chemical Analysis

Specimens were heated at 65°C for 72 h, and dried materials were ground for chemical analysis. The contents of dry matter (DM), crude protein (CP), water-soluble carbohydrates (WSC), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were examined using previously established methods (He et al., 2020c). The contents of DM, CP, WSC, NDF and ADF in stylo before ensiled were 30.10, 10.49, 1.23, 51.04, and 40.76%, respectively. The fermentation quality of silage was determined using distilled water extracts. Briefly, 50 g wet silage was blended with 200 mL distilled water, followed by incubation at 4°C for 24 h and then filtration for analyzing. The pH and contents of lactic acid, acetic acid, propionic acid, butyric acid and ammonia-N were determined using previously established methods (Li et al., 2019).

Microbial Diversity Analysis

DNA Isolation and 16S rRNA Gene Sequencing

The above-mentioned extracts were used for the molecular analysis of the microbiota. Microbial DNA was isolated from silage specimens with the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, United States) according to manufacturer's instructions. The concentration and purity of extracted DNA were assessed by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States), and DNA integrity was confirmed by electrophoresis on 1% agarose gel. Primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and

806R (5'-GGACTACHVGGGTWTCTAAT-3') were adopted to amplify the V3–V4 hypervariable regions of the bacterial 16S rRNA gene using thermocycler PCR system (GeneAmp 9700, ABI, United States). After PCR products were purified and quantified, next-generation sequencing was carried out using Illumina MiSeq 2500 platform (Illumina, Inc., San Diego, CA, United States), and paired-end reads of 250 bp were generated.

Processing and Analysis of Sequencing Data

The assembly of tags was carried out using filtered reads according to the principles as follows: overlap between paired-end reads should be more than 10-bp overlap and less than 2% mismatch. The unique tags were obtained by removing redundant tags using software MOTHUR (Schloss et al., 2009). The abundance was then determined using the resultant unique tags. The high-quality reads were grouped into operational taxonomic units (OTUs) defined at a similarity of 97%. Diversity metrics were determined using the core-diversity plug-in within QIIME2[1] (Callahan et al., 2016). The microbial diversity within an individual sample was assessed using the alpha diversity indices, including observed OTUs, Chao1 richness estimator, Shannon diversity index, Simpson and ACE index. Beta diversity was analyzed to assess the structural variation of microbiota across specimens, and then principal component analysis (PCA) was determined (Vázquez-Baeza et al., 2013). The described methods were employed to identify the bacterial strains with different abundances among samples and groups (Segata et al., 2011). Unless specified above, parameters used in the analysis were set as default. The sequencing data were deposited in the Sequence Read Archive (SRA) under the accession number of PRJNA629094.

Metabolite Analysis

Metabolite Extraction

Briefly, 100 μL sample was placed into a 1.5-mL tube, and 350 μL pre-cold methanol and 10 μL internal standard (L-2-chlorophenylalanine, 1 mg/mL stock) were added into the tube, followed by vortex mixing for 30 s. The mixture was subjected to ultrasonication for 10 min in ice water and then centrifuged at 12,000 *g* for 15 min at 4°C. Next, 100 μL supernatant was collected and placed into a new tube. To prepare the QC (quality control) sample, 70 μL of each sample was collected, pooled and evaporated in a vacuum concentrator. Subsequently, 40 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) was added, followed by incubation at 80°C for 30 min, and then the mixture was derivatized by 50 μL of BSTFA regent (1% TMCS, v/v) at 70°C for 1.5 h. The sample was gradually cooled to room temperature, and 5 μL of FAMEs (in chloroform) was added to QC sample. All specimens were then subjected to gas chromatograph coupled with a time-of-flight mass spectrometer (GC-TOF-MS).

GC-TOF-MS Analysis

An Agilent 7,890 gas chromatograph coupled with a time-of-flight mass spectrometer was adopted for GC-TOF-MS analysis, and a DB-5MS capillary column was employed in such system. Briefly, 1 μL aliquot of specimen was injected in splitless mode. Helium was employed as the carrier gas, the front inlet purge flow was set at 3 mL min^{-1}, and the gas flow rate through the column was set at 1 mL min^{-1}. The initial temperature was maintained at 50°C for 1 min, and then it was raised to 310°C at a rate of 10°C min^{-1} and kept at 310°C for 8 min. The temperatures of injection, transfer line, and ion source were set at 280, 280, and 250°C, respectively. The energy was −70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode within the m/z range of 50–500 at a rate of 12.5 spectra per second after a solvent delay of 6.25 min.

Data Preprocessing and Annotation

Raw data analysis, including peak extraction, baseline adjustment, deconvolution, alignment and integration, was completed with Chroma TOF (V 4.3x, LECO) software, and LECO-Fiehn Rtx5 database was adopted to identify metabolites by matching the mass spectrum and retention index. Finally, the peaks found in less than half of QC samples or RSD > 30% in QC samples were eliminated.

Metabolite Data Analysis

The metabolite data were analyzed according to previous work (Ren et al., 2018; Xu et al., 2019). All specimens were tested using PCA and PLS-DA models. Differentially expressed metabolites (DEMs) ($P < 0.05$) were identified using the OPLS-DA model with first principal-component of VIP (variable importance in the projection) values (VIP > 1) in combination with Student's *t*-test. Pearson correlation coefficients were calculated to examine the correlation between metabolite and relative abundance of microbial groups. The heatmap was generated by the corrplot package in R and performed using BMKCloud[2].

Statistics

The impacts of application rate were investigated using one-way analysis of variance in SAS 9.3 software (SAS Institute Inc., Cary, NC, United States). Duncan's multiple range test was adopted to identify significant differences, and $P < 0.05$ was considered statistically significant.

RESULTS

Fermentation Quality of Stylo Silage Treated With Melatonin

Table 1 illustrates the fermentation characteristics of stylo silage after fermentation. The pH of melatonin-treated silages was reduced compared with the CK group, and the lowest ($P < 0.05$) pH value was observed in the Mela1 group. Melatonin treatments remarkably ($P < 0.05$) elevated lactic acid contents, and the lowest lactic acid level was found in the CK group ($P < 0.05$), while there was no obvious difference among melatonin-treated groups. The acetic acid contents were no significant difference was detected ($P > 0.05$). Melatonin treatments dramatically

[1]https://docs.qiime2.org/2019.1/

[2]http://www.biocloud.net/

TABLE 1 | Fermentation quality of Stylo silage supplemented with melatonin.

	pH	Lactic acid (% DM)	Acetic acid (% DM)	Propionic acid (% DM)	Butyric acid (% DM)	Total acid (% DM)	NH_3-N (% DM)
CK	4.53 ± 0.04^a	1.72 ± 0.13^b	3.70 ± 0.27^a	0.16 ± 0.03^a	0.08 ± 0.03^a	5.66 ± 0.25^b	5.27±^{a}
Mela1	4.20 ± 0.05^b	5.92 ± 0.24^a	3.32 ± 0.37^a	0.06 ± 0.06^b	0.00	9.30 ± 0.42^a	4.11±^{b}
Mela2	4.21 ± 0.02^b	6.04 ± 0.13^a	4.05 ± 0.44^a	0.04 ± 0.01^b	0.00	10.13 ± 0.44^a	4.08±^{b}
Mela3	4.27 ± 0.04^b	6.12 ± 0.29^a	3.10 ± 0.22^a	0.06 ± 0.01^b	0.00	9.28 ± 0.17^a	4.09±^{b}

CK, control; Mela1, 5 mg/kg Melatonin; Mela2, 10 mg/kg Melatonin; Mela3, 20 mg/kg Melatonin. Means within the same column with different letters are significantly different ($P < 0.05$).

($P < 0.05$) reduced the levels of propionic acid and butyric acid, and their highest levels were both detected in the CK group ($P < 0.05$). Furthermore, no butyric acid was detected in three melatonin-treated groups. Melatonin treatments remarkably ($P < 0.05$) elevated total acid contents, and the lowest level was detected in the CK group ($P < 0.05$), while three melatonin-treated groups had no significant difference ($P > 0.05$). In addition, melatonin dramatically ($P < 0.05$) reduced the levels of NH_3-N, and the highest levels was detected in the CK group ($P < 0.05$). The above-mentioned results revealed that the melatonin treatments could promote the fermentation quality, and there was no dramatic difference among melatonin-treated silages.

Microbiota Community of Stylo Silage Treated With Melatonin

A total of 913,107 raw reads and 883,046 raw tags were generated. After sequencing data were processed, averagely 73, 489 clean tags and 64,108 effective tags were obtained in each silage sample.

Figure 1 shows the α-diversity of the bacterial community of silages. Melatonin treatment affected the Ace, Chao 1, Shannon and Simpson indices of microbial diversity and richness (**Figure 1**). The richness indices (Chao 1 and ACE) were not significantly different between the CK and Mela3 groups, while they were significantly higher compared with the Mela1 and Mela2 groups ($P < 0.05$). These indices showed that melatonin decreased the richness. In contrast to the richness indices, however, the Shannon index was higher, and the Simpson index in the additive-treated groups was lower than that in the CK group ($P < 0.05$), suggesting that melatonin treatments resulted in a higher diversity. Generally speaking, the high OTU number, Shannon index, as well as the low Simpson index indicate a higher microbial abundance and a higher diversity. The PCA and cluster tree were employed to examine the correlations among the community structures of the silage microbial community. A clear separation and difference of bacterial communities were found in four groups (**Figure 2A**), the CK and Mela3 groups had further genetic distance compared with the Mela1 and Mela2 groups (**Figure 2B**), and these results suggested that the microbial composition was changed in the silage process due to melatonin supplementation. Therefore, we drew a conclusion based on the α-diversity and β-diversity that the melatonin supplementation could affect the microbial diversity and community structure of stylo silage.

Figure 3A1,2 describes the microbial community at the phylum level. *Proteobacteria*, *Firmicutes*, *Bacteroidetes,* and *Actinobacteria* were the dominant phyla in all groups. The community was shifted along with the melatonin treatments (**Figure 3A3**), the abundances of *Proteobacteria*, *Bacteroidetes,* and *Actinobacteria* were increased, while the abundance of *Firmicutes* was decreased in additive-treated groups compared with the CK group ($P < 0.05$). To further investigate the effects of additives on microbial community during ensiling, we examined the bacterial structures of stylo silage at the genus level (**Figure 3B1,2**). *Weissella*, *Enterobacter*, *Pantoea*, *Stenotrophomonas*, and *Sphingobacterium* were the predominant genera in the four groups. The abundance of *Weissella* was decreased along with the ensiling process (**Figure 3B3**), while the abundances of *Enterobacter*, *Pantoea*, *Stenotrophomonas* and *Sphingobacterium*, *Pseudomonas*, *Acinetobacter*, *Brevundimonas,* and *Lactobacillus* were increased (except for *Enterobacter* in the Mela3 group). In addition, the number of unclassified species was obviously increased from 14.09% (CK) to 40.17% (Mela3). The differences in microbial community among groups were detected using the linear discriminant analysis (LDA) effect size (LEfSe) method, and the specific bacterial flora in each group was explored (LDA score > 4.0). **Figures 4A,B** shows that melatonin exerted a dramatic impact on the microbial community. Melatonin supplementation significantly elevated the relative abundance of *Pseudomonadaceae*, and reduced the abundance of *Weissella*. *Weissella* was the most abundant genus in the CK group, and *Pseudomonadaceae* was most abundant family in the Mela3 group, which could be identified as the specific bacterial taxa associated with melatonin.

Moreover, we showed that melatonin shaped the silage microbiota, including an increase in Shannon index but a decrease in Simpson index, implying that melatonin treatment could raise the microbial diversity. Meanwhile, the β-diversity analysis found distinct differences in microbia composition between the CK and melatonin-treated groups, and such differences were further amplified when the amount of added melatonin was increased. In addition, melatonin changed the microbial composition of stylo silage, the abundance of *Weissella* was decreased, and the abundances of unclassified species were increased along with the melatonin treatment.

Metabolism of Stylo Silages Treated With Melatonin

To clarify the impacts of melatonin addition on silage microbiota, we further determined the levels of metabolites in the silage specimens among four groups. According to PCA (3D), the metabolites in four groups of specimens were obviously

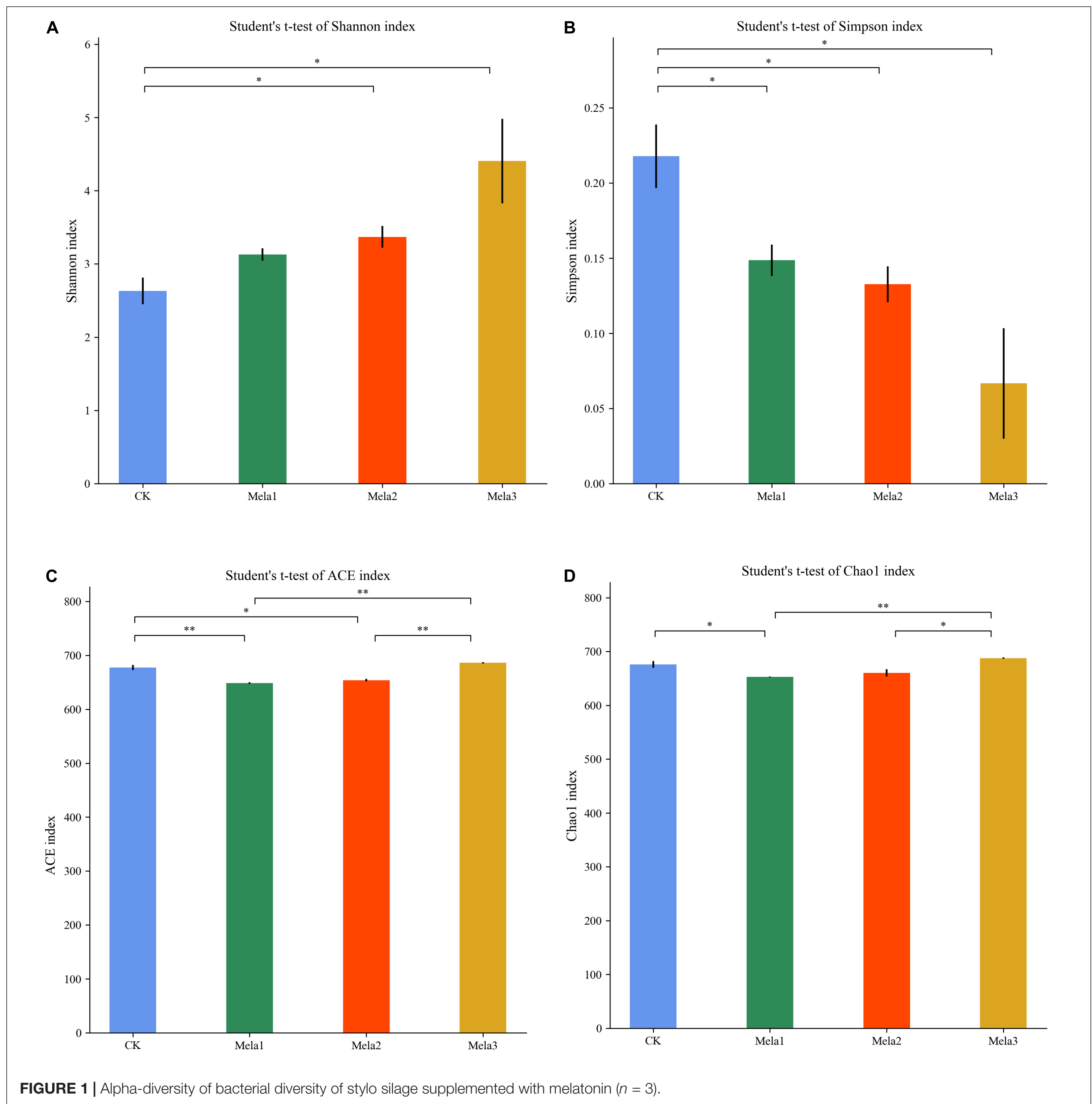

FIGURE 1 | Alpha-diversity of bacterial diversity of stylo silage supplemented with melatonin (n = 3).

segregated by PC1, PC2 and PC3, representing 13.82, 12.23, and 11.18% of variations among specimens with various additives, respectively (**Figure 5**). To analyze metabolites in the silage specimens, 214 metabolites were found from 500 peaks in the chromatograms, and 64 known metabolites were identified. These identified metabolites were divided into 21 categories, including carboxylic acids and derivatives, organooxygen compounds, benzene and substituted derivatives, fatty acyls, hydroxy acids and derivatives, cinnamic acids and derivatives, diazines, imidazopyrimidines, non-metal oxoanionic compounds, phenols, azoles, heteroaromatic compounds, indoles and derivatives, keto acids and derivatives, lactams, naphthalenes, organonitrogen compounds, phenylpropanoic acids, prenol lipids, pyrimidine nucleosides, and tropane alkaloids (**Figure 6D**).

Table 2 and **Figures 6A–C** list the identified potential markers. When the three melatonin-treated groups were compared with the CK group, the contents of 10 metabolites were significantly changed between the CK and Mela1 groups. For example, the contents of sulfuric acid (P = 0.019), 2-deoxyuridine

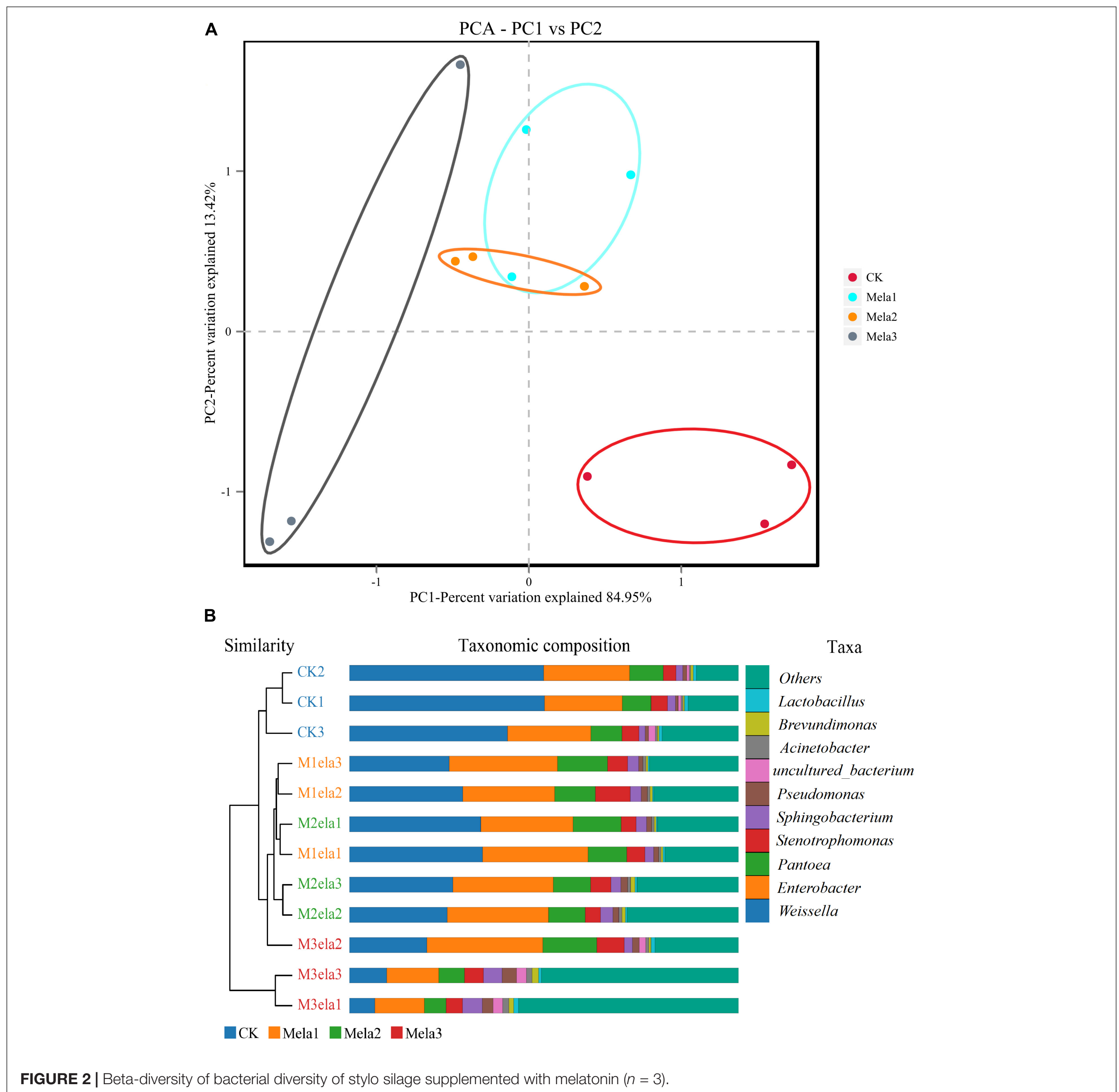

FIGURE 2 | Beta-diversity of bacterial diversity of stylo silage supplemented with melatonin (n = 3).

(P = 0.049), 4-methylbenzyl alcohol (P = 0.04), thymine (P = 0.018), L-malic acid (P = 0.031), 4-hydroxy-6-methyl-2-pyrone (P = 0.041), allose (P = 0.038), creatine degr (P = 0.05), and 2-deoxy-D-glucose (P = 0.035) were down-regulated, while the content of alpha-D-glucosamine 1-phosphate (P = 0.018) was up-regulated. Moreover, the contents of nine metabolites were significantly altered between the CK and Mela2 groups. For example, the contents of malonic acid (P = 0.032), carbobenzyloxy-L-leucine degr (P = 0.036), glycine (P = 0.005), threonine (P = 0.001), methionine (P = 0.038), methionine sulfoxide (P = 0.008), 2-deoxy-D-galactose (P = 0.019), ornithine (P = 0.028), and 2,6-diaminopimelic acid (P = 0.034) were up-regulated. In addition, the content of one metabolite, sulfuric acid (P = 0.023), was significantly down-regulated between the CK and Mela3 groups. KEGG assay indicated that melatonin significantly changed the microbial functions, such as pyrimidine metabolism, monobactam biosynthesis, amino sugar and nucleotide sugar metabolism, sulfur metabolism, citrate cycle (TCA cycle), purine metabolism, cysteine and methionine metabolism, carbon fixation in photosynthetic organisms, pyruvate metabolism, carbon metabolism, glyoxylate and dicarboxylate metabolism and ABC transporters between the CK and Mela1 groups

FIGURE 3 | The bacterial abundance at the phylum and genus levels in stylo silage supplemented with melatonin (n = 3).

(**Figure 6E**). The monobactam biosynthesis, sulfur metabolism, purine metabolism, cysteine and methionine metabolism and ABC transporters were also altered between the CK and Mela3 groups (**Figure 6F**). Collectively, melatonin addition significantly altered the metabolism of silage microbiota.

Correlations Between the Relative Abundances of Bacteria and Metabolites of Stylo Silages

We also assessed the correlation between the metabolites and microbiome of stylo silages. At the genus level, a Spearman correlation heatmap was created for the microbial community within the silages (**Figure 7**). Alpha-D-glucosamine-1-phosphate was positively associated with *Sphingopyxis* and *Candidatus Saccharimonas*, while it was negatively associated with *Lactobacillus*. Sulfuric acid was negatively associated with *Candidatus Saccharimonas*, *Atopostipes*, *Alcaligenes*, *Sphingopyxis,* and *Parapedobacter*. Moreover, 2-deoxy-D-glucose, thymine and 4-hydroxy-6-methyl-2-pyrone were positively correlated with uncultured_bacterium_f_*Gemmatimonadaceae*, while they were negatively correlated with *Petrimonas*, *Atopostipes*, *Alcaligenes*, *Ruminococcaceae*_UCG-005, and *Parapedobacter*. In addition, 4-methylbenzyl alcohol, 2-deoxyuridine and creatine degr were positively correlated with uncultured_bacterium_f_*Gemmatimonadaceae*, while they were negatively correlated with *Petrimonas* and *Ruminococcaceae*_UCG-005. L-Malic acid was negatively associated with *Candidatus, Saccharimonas,* and *Parapedobacter*. This study showed significant correlation between many different bacteria and the metabolites of the stylo silage. A great deal of metabolites were positively associated with LAB species, but negatively associated with undesirable bacteria during ensiling. Silage fermentation is a very complex biological process, which involves a large variety of microorganisms. For instance, the process produces many different metabolites, which can determine the fermentation quality.

DISCUSSION

Melatonin Improves Fermentation Quality of Stylo Silage

Melatonin is widely used in postharvest storage of fruits, which can enhance the antioxidant activity and effectively retard the decline of fruit quality (Gao et al., 2016; Hu et al., 2017; Liu et al., 2018; Zhai et al., 2018; Zhang et al., 2018). In the present study, we showed that melatonin facilitated the silage quality of stylo through the following fermentation characteristics. The pH value of additive-treated silages was close to 4.2, ensuring the well preservation of stylo silage (Edwards and McDonald, 1978).

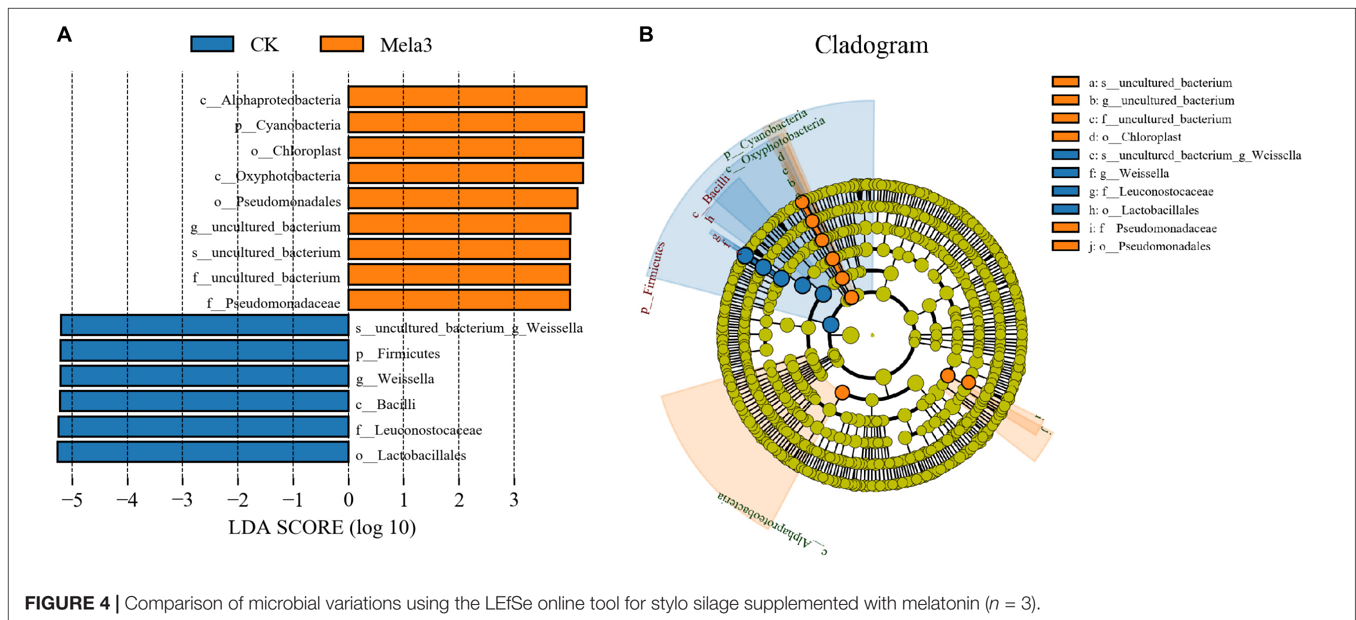

FIGURE 4 | Comparison of microbial variations using the LEfSe online tool for stylo silage supplemented with melatonin (*n* = 3).

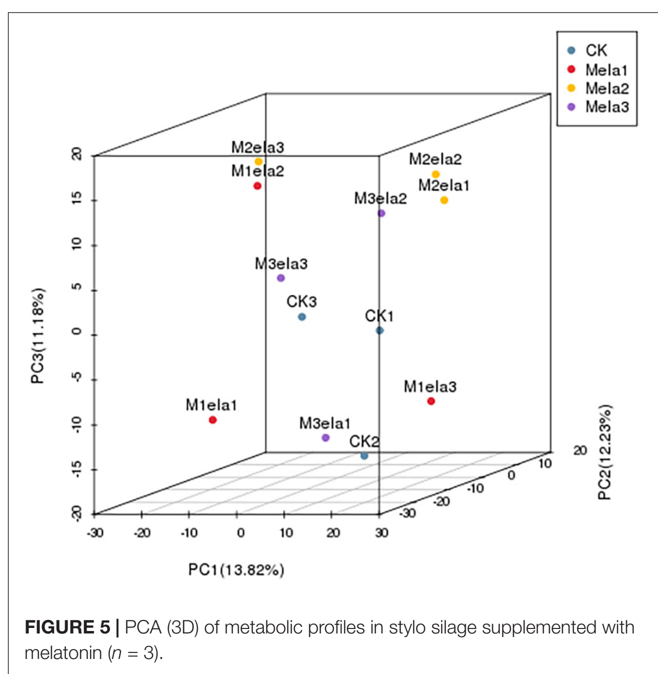

FIGURE 5 | PCA (3D) of metabolic profiles in stylo silage supplemented with melatonin (*n* = 3).

A previous study has shown higher pH values at 5.0 or above in stylo silage, which is maybe caused melatonin treatment more efficient than *Moringa oleifera* leaves or gallic acid addition (He et al., 2020a,c). Lactic acid in silage is the dominant product from fermentation, which is an important index to evaluate silage quality. In the present study, melatonin promote the production of lactic acid, which increased 244.18–255.81% compare with control group, and the results were similar with other additives (Li et al., 2017; He et al., 2020a). In the current study, the level of butyric acid was not detectable in melatonin-treated silages, because melatonin has the effects of antioxidation and inhibition of undesirable bacteria, there were no *Clostridium* in the silage bacterial communities, indicating that the stylo was well preserved. Similar studies have been reported, (He et al., 2020a,b) reported that butyric acid was not detected in silage after adding gallic acid or tannic acid, thus improving the fermentation quality of silage. Gallic acid and tannic acid are polyphenolic antioxidants, which have antifungal and antiviral effects, and can inhibit the harmful microorganisms such as *Clostridium*, *Listeria,* and *Escherichia coli* in the process of silage (He et al., 2020a,b). The total acid content showed similar trend as lactic acid and acetic acid, while melatonin-treated silage improved total acid content 63.95–78.97% compared with the CK group after ensiling. In this study, melatonin treatment reduced the content of NH_3-N of control stylo silage, the effect similar with gallic acid (He et al., 2020a). However, the NH_3-N contents of stylo silage were various. One study reported the NH_3-N in stylo and *Moringa oleifera* leaves mixed silage were range from 5.07 to 11.1%(He et al., 2020c). In contrary, gallic acid reduced NH_3-N from 1.46 to 0.29% (He et al., 2020a). These differences may be caused by the different antioxidant or antibacterial ability of additives. These results indicated that melatonin addition promoted the fermentation quality, and the addition of various amounts of melatonin in this study showed similarly effect. Therefore, it is suggested that melatonin with a minimum dosage (5 mg/kg) can achieve the effect.

Melatonin Addition Shapes Microbiota Community of Stylo Silage

In present study, the beta-diversity shown separation and difference of bacterial communities of stylo silage. These results suggested that the microbial composition was changed in the silage process due to melatonin treatments. The specific changes were reflected by the differences in bacterial composition at the genus level among different treatments stylo silage. Some

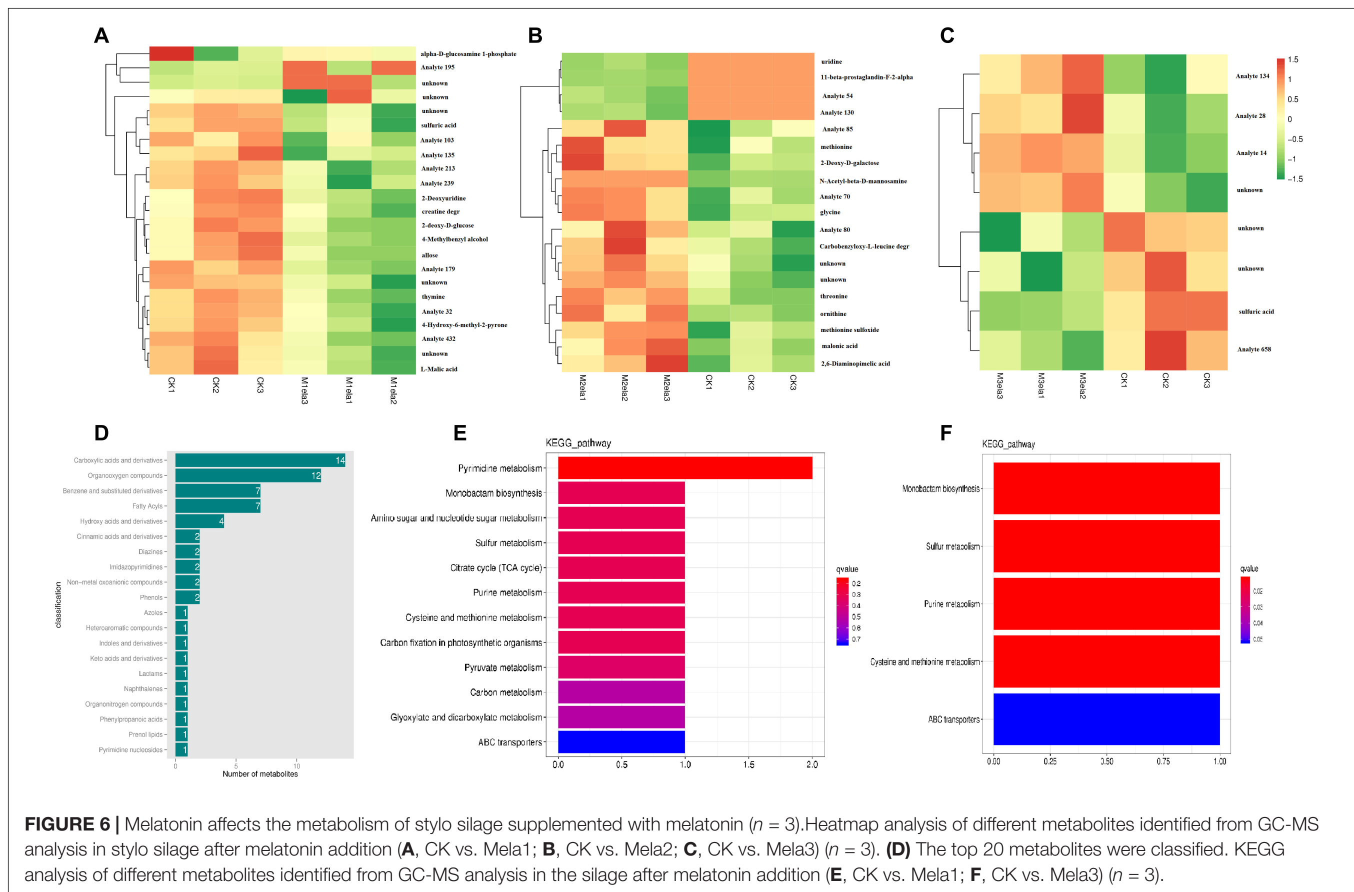

FIGURE 6 | Melatonin affects the metabolism of stylo silage supplemented with melatonin (n = 3).Heatmap analysis of different metabolites identified from GC-MS analysis in stylo silage after melatonin addition (**A**, CK vs. Mela1; **B**, CK vs. Mela2; **C**, CK vs. Mela3) (n = 3). **(D)** The top 20 metabolites were classified. KEGG analysis of different metabolites identified from GC-MS analysis in the silage after melatonin addition (**E**, CK vs. Mela1; **F**, CK vs. Mela3) (n = 3).

previous reports consistent with this study, additives such as *Moringa oleifera powder* and gallic acid shaped the bacterial community structure (He et al., 2020a,c). In particular, the structure of the bacterial communities greatly at the genus level. As a lactic acid-producing bacterial strain, *Weissella* exists widely in fermented food or silage (Cai et al., 1998). Furthermore, it is the dominant group at the beginning of silage fermentation before it is replaced by other lactic acid-producing microorganisms after ensiling (Cai et al., 1998; Guan et al., 2018; Ding et al., 2020). This might be explained by the fact that the fermentation capacity of *Weissella* was weaker compared with other microorganisms, leading to weakened competitiveness and its replacement by *Pantoea*, *Stenotrophomonas*, *Sphingobacterium*, *Pseudomonas*, *Acinetobacter*, *Brevundimonas,* and *Lactobacillus*. *Enterobacter* is one of the major undesirable microorganisms in silage that produce acetic acid to raise the pH, which, as a result, reduces the fermentation quality (Ni et al., 2017; Wang et al., 2019; Lv et al., 2020). In this study, the abundance of *Enterobacter* was higher and similar in four groups, which was reflected by the relatively high level of acetic acid. Therefore, the effect of *Enterobacter* on stylo fermentation quality was slight. *Pantoea* (8–11.9%) was the third dominant strain in the present study, which was higher than *A. villosum* silage (1.44–4.56%) (Lv et al., 2020). Its effect on silage fermentation still has a dispute. Ogunade et al. (2018) have reported that *Pantoea* is negatively correlated with ammonia-N, and *Pantoea* is beneficial to silage fermentation. However, some studies have claimed that *Pantoea* is an undesirable microbial strain because it competes the fermentation substrate with LAB[22]. *Stenotrophomonas* and *Sphingobacterium* belong to aerobic or facultative anaerobic non-fermentative Gram-negative bacilli, which can utilize a variety of sugars and produce acid (Yabuuchi et al., 1983; Palleroni and Bradbury, 1993). *Pseudomonas* is considered as an undesirable bacterial strain for silage due to its possibility of biogenic amine production as well as decreased protein content and nutritional value (Santos Silla, 1996; Dunière et al., 2013). Ogunade et al. (2018) found that these four microorganisms were negatively correlated with pH, ammonium nitrogen, yeast and mold, suggesting that they may be beneficial to silage fermentation. Moreover, in this study, *Pantoea*, *Stenotrophomonas*, *Sphingobacterium, and Pseudomonas* have relative lower abundances, and which significantly increased in melatonin-treated groups. Therefore, the underlying mechanisms need to be further investigated. We speculated that the increased abundances of these microbial species enhanced the biodiversity of the silage micro-ecosystem, while increased the quantity and abundance of lactic acid producing microorganisms. These makes the silage micro-ecosystem more species-rich and conducive to the dynamic balance of beneficial and undesirable microorganisms, and then generated beneficial synergistic effect, leading to improved silage quality. Another possibility could be attributed to the

TABLE 2 | Candidate silage metabolites that were different between the CK and melatonin-treated groups.

Compounds	Fold_change	*P*-value	VIP
CK_vs._Mela1			
Sulfuric acid	0.604	0.019	1.664
2-Deoxyuridine	0.709	0.049	1.557
4-Methylbenzyl alcohol	0.704	0.04	1.611
Thymine	0.692	0.018	1.687
L-Malic acid	0.726	0.031	1.58
4-Hydroxy-6-methyl-2-pyrone	0.729	0.041	1.633
Allose 1	0.658	0.038	1.568
Creatine degr	0.754	0.05	1.498
2-Deoxy-D-glucose 1	0.654	0.035	1.577
Alpha-D-glucosamine 1-phosphate	4.666	0.018	1.639
CK_vs._Mela2			
Malonic acid 1	1.296	0.032	1.998
Carbobenzyloxy-L-leucine degr1	1.29	0.036	1.984
Glycine 2	1.488	0.005	2.082
Threonine 1	1.611	0.001	2.197
Methionine 1	1.33	0.038	1.807
Methionine sulfoxide 2	1.488	0.008	2.075
2-Deoxy-D-galactose 2	1.293	0.019	1.991
Ornithine 1	1.666	0.028	2.072
2,6-Diaminopimelic acid 2	1.4	0.034	1.949
CK_vs._Mela3			
Sulfuric acid	0.715	0.023	2.116

CK, control; Mela1, 5 mg/kg Melatonin; Mela2, 10 mg/kg Melatonin; Mela3, 20 mg/kg Melatonin. Fold change (fold change > 1 means melatonin < control; fold change < 1 means melatonin > control). N = 3, the data were analyzed by unpaired t-test. P-value, t-test significance. VIP, variable importance in the projection.

increased number of unclassified microorganisms that affect the fermentation of silage. However, in-depth investigations are required to clearly understand their roles in silage fermentation. Therefore, the effects of melatonin treatment on microbial composition need to be further explored using Single Molecule, Real-Time (SMRT) Sequencing or metagenomic sequencing.

Melatonin Addition Alters the Metabolism of Stylo Silages

The fermentation process of silage is complex, involving the interaction of many microorganisms mainly composed of lactic acid bacteria and their metabolites. Besides its effects on the composition of silage microbiota, melatonin also changed the metabolism of silage microbiota. For a long time, the studies on silage metabolism are few, and the type and quantity of metabolites in silage remain largely unclear. The currently available research mainly focuses on metabolites related to fermentation quality and aerobic stability, such as lactic acid, acetic acid, propionic acid, butyric acid and propanediol (Broberg et al., 2007). The impacts of additives on the silage metabolites are still unknown. Guo et al. (2018) have reported the metabolites in alfalfa silage, showing that the LAB treatments raise the desirable compounds of 2,3-butandiol, adenine and amino acids, but decrease the content of undesirable cadaverine, which can be attributed to the role of corruption, leading to bad fermentation quality. Xu et al. (2019) have found that there are more amino acids (phenylalanine, lysine, tyrosine and glycine), phenolic acids (4-hydroxycinnamic acid and 3,4-dihydroxycinnamic acid), flavoring agent (gluconic lactone) and organic acids (lauric acid, 3-hydroxypropionic acid, pentadecanoic acid, oxamic acid and isocitric acid) in whole crop corn silage treated with LAB. In the present study, melatonin supplementation up-regulated the contents of alpha-D-glucosamine-1-phosphate, malonic acid, carbobenzyloxy-L-leucine degr, glycine, threonine, methionine, methionine sulfoxide, 2-deoxy-D-galactose, ornithine and 2,6-diaminopimelic acid, while such supplementation down-regulated the contents of sulfuric acid, 2-deoxyuridine, 4-methylbenzyl alcohol, thymine, L-malic acid, 4-hydroxy-6-methyl-2-pyrone, allose, creatine degr and 2-deoxy-D-glucose. The increase of amino acid content was consistent with previous reports (Guo et al., 2018; Xu et al., 2019). Malonic acid is the intermediate for the production of vitamins and amino acids. These metabolites with beneficial effects could also be considered as an evaluation index for fermentation quality. The reduced metabolites, such as nucleic acid metabolites, and special sugars could be used as biomarkers of poor fermentation quality in stylo silage. The similar phenomenon has been reported in alfalfa silage that cadaverine suggests bad fermentation quality (Guo et al., 2018). Different metabolites affected various metabolic pathways, including pyrimidine metabolism, monobactam biosynthesis, amino sugar and nucleotide sugar metabolism, sulfur metabolism, citrate cycle, purine metabolism, cysteine and methionine metabolism, carbon fixation in photosynthetic organisms, pyruvate metabolism, carbon metabolism, glyoxylate and dicarboxylate metabolism and ABC transporters. In general, the addition of melatonin in stylo silage changed the composition of metabolites, increased the beneficial metabolites and promoted the silage fermentation quality. Nevertheless, it remains unclear whether the altered metabolites are merely the effect of melatonin on silage microbiota, or whether melatonin modulates the fermentation quality via these metabolites. Therefore, it is necessary to explore the mechanisms underlying the melatonin-induced changes in fermentation quality.

Spearman correlation analysis was carried out to explore the correlations between microbial and metabolites. Xu et al. (2019) have found that metabolites in corn silage are positively associated with LAB species but negatively associated with undesirable microorganisms. In this study, alpha-D-glucosamine-1-phosphate was phosphate derivatives of glucose, and the result reflected the ability of glucose utilization to some extent. Lactobacillus had a stronger ability than *Sphingopyxis* and *Candidatus Saccharimonas*, leading to the opposite correlation. Acidic environment will hinder the growth of pathogen and spoilage, which is negatively correlated with sulfuric acid. In addition, 2-deoxy-D-glucose and thymine can be used by bacteria through oxidation or fermentation. Therefore, they were negatively correlated. L-Malic acid was negatively associated with *Candidatus Saccharimonas* and *Parapedobacter*, because the lower pH could inhibit their growth. Generally, there were significant correlations between many different bacteria and the metabolites of the stylo silage, and it was mainly determined by the characteristics and functions of metabolites. Furthermore,

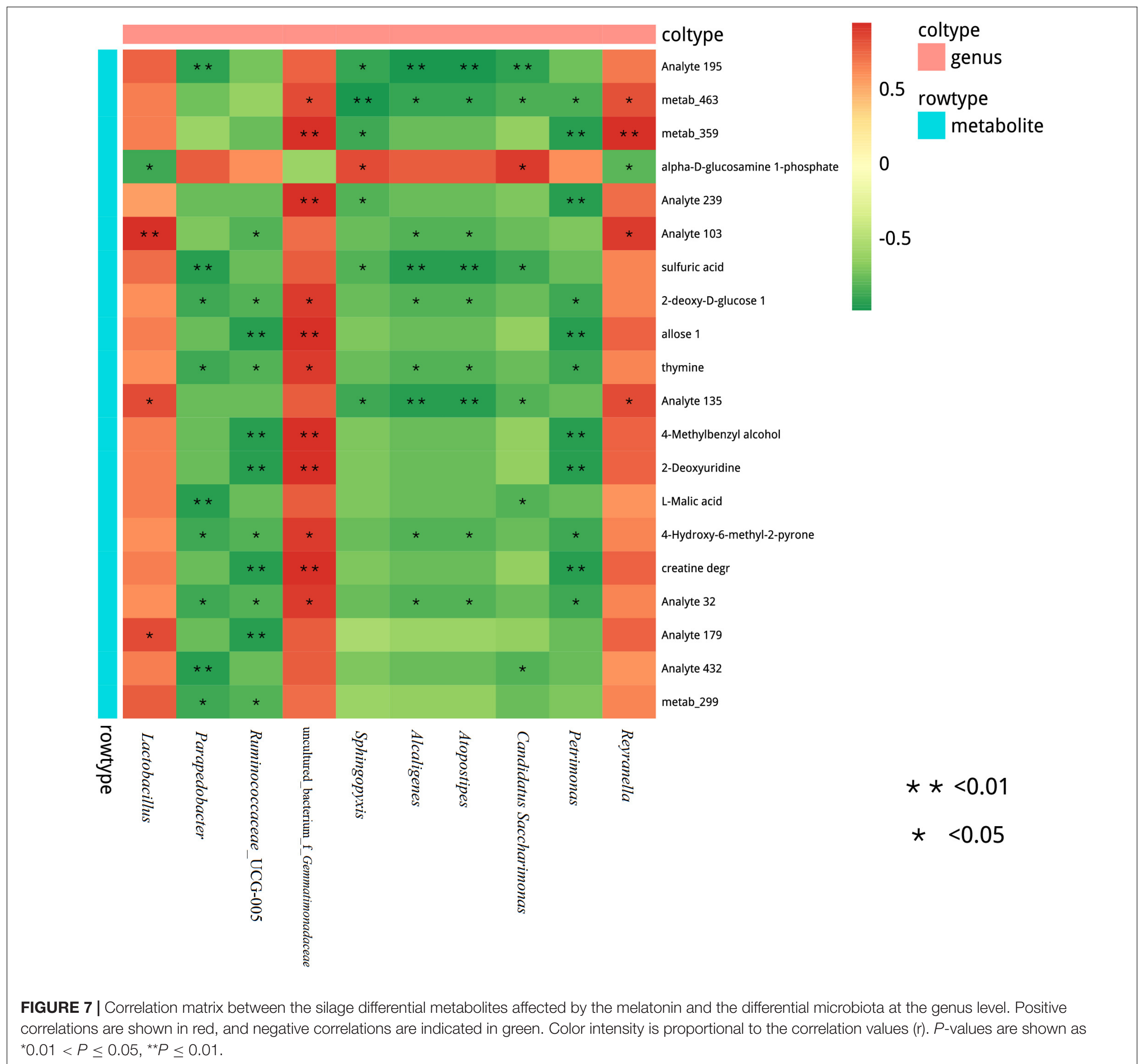

FIGURE 7 | Correlation matrix between the silage differential metabolites affected by the melatonin and the differential microbiota at the genus level. Positive correlations are shown in red, and negative correlations are indicated in green. Color intensity is proportional to the correlation values (r). *P*-values are shown as *$0.01 < P \leq 0.05$, **$P \leq 0.01$.

fermentation process produces many different metabolites, which can determine the fermentation quality. It is worth noting that the metabolites with biological functions are negatively correlated with some lactic acid bacteria (species level) in silage, which may be due to the low concentration of metabolites in the sample, which cannot be accurately detected, but the low content of metabolites may still affect the silage quality and even the health of animals. Therefore, it is still of great significance to study the correlation between microorganisms and metabolites in stylo silage, which can be used to screen targeted additives to regulate silage fermentation and prepare high-quality silage. It is important to note that correlation is not causation, it is based on statistical data, the parameters are related, the results are only speculation. Nevertheless, it is of great significance to study the correlation between stylo silage microorganisms and metabolites with biological functions, which can provide a theoretical basis for screening targeted additives to regulate silage fermentation and prepare high-quality silage.

In summary, melatonin addition increased the quantity and abundance of lactic acid producing microorganisms, while raised the beneficial metabolites malonic acid and amino acid and reduced some nucleic acid and special sugars, and then affected the pathway of amino acid metabolism, nucleic acid metabolism and carbon metabolism etc. Finally, it inhibited undesirable microorganisms and raised the lactic acid content and dropped pH, improved the silage fermentation quality.

CONCLUSION

Melatonin addition significantly improved silage quality of stylo, and the lowest addition (5 mg/kg) showed the beneficial effect, including the increased contents of lactic acid and total acid, as well as the decrease in pH and butyric acid. Additionally, besides its effects on enhancing the micrbial diversity (such as the increase in shannon indices but the decrease in simpson indices), melatonin significantly shaped the composition of silage microbiota (such as the increased abundances of *Pantoea*, *Stenotrophomonas*, *Sphingobacterium,* and *Pseudomonas*, and the decreased abundance of *Weissella*), and the metabolism of silage microbiota. Moreover, Melatonin addition also dramatically affected the metabolites of sylo silage, such as raised malonic acid and some amino acid metabolism(glycine, threonine, methionine and ornithine), while reduced nucleic acid metabolism(2-deoxyuridine and thymine) and carbon metabolism(allose and 2-deoxy-D-glucose). Therefore, the analysis of microbiome and metabonomics of stylo silage can improve our understanding of the biological process of silage fermentation, and it is also conducive to scientifically evaluate and regulate the silage quality.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The sequencing data were deposited in the Sequence Read Archive (SRA) under the accession number of PRJNA629094.

AUTHOR CONTRIBUTIONS

ML, XZ, and JT did the experimental design work. ML, XZ, RL, LZ, and JT conducted the experiments. ML, XZ, RL, LZ, JT, and HZ analyzed the data. ML and XZ wrote the manuscript. All authors read and approved the manuscript.

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Lentilactobacillus hilgardii Inoculum, Dry Matter Contents at Harvest and Length of Conservation Affect Fermentation Characteristics and Aerobic Stability of Corn Silage

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Heterofermentative *Lentilactobacillus hilgardii* isolated from sugarcane silage, has recently been proposed as a silage inoculant to increase aerobic stability. Various conditions can influence the activity of LAB and their ability to alter silage quality (e.g., DM content and length of conservation). The aim of this study has been to evaluate the effect of *L. hilgardii* on the fermentation quality and aerobic stability of whole crop corn silage with different DM contents (from 26 to 45%), conserved for various conservation lengths (13–272 days). The silages were analyzed for their DM content, pH, fermentative profile, microbial count, and aerobic stability. *L. hilgardii* showed a positive effect on improving the aerobic stability of silages, due its ability to produce acetic acid, and reduced the yeast count. The acetic acid content increased as the conservation period increased and decreased as the DM content increased. The yeast count was reduced during conservation in a DM dependent manner and the inoculation with LH determined a reduction in the count of 0.48 log cfu/g. The aerobic stability increased as the conservation period increased, and the treatment with LH on average increased the aerobic stability by 19 h. The results of this experiment suggest that higher aerobic stability could be achieved in corn silages by ensiling at medium or low DM contents, or by increasing the length of conservation if a higher DM content at ensiling is needed. The inoculation with LH helps to improve the aerobic stability of corn silages by reducing the yeast count.

Keywords: dry matter, inocula, yeast count, conservation time, aerobic stability

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INTRODUCTION

The presence of spoiled silages on a farm is a challenge, and it results in a reduction of the nutritive value of the silage and an increase in the risks to animals and humans (Driehuis et al., 2018). During the feed-out phase, the contact of the mass with air stimulates the growth of the aerobic microorganisms that are most often responsible for the onset of aerobic instability (Borreani et al., 2018). Aerobic deterioration is common in many silages that are opened and exposed to air, with the rate of spoilage being highly dependent on the numbers and activity of the spoilage organisms in the silage and on the fermentative profile of the silo at opening (Muck et al., 2018). It is generall

accepted that yeasts play the major role in initiating aerobic spoilage (Pahlow et al., 2003) and the aerobic stability of corn silage has been found to be closely correlated with the number of yeasts (Borreani et al., 2018). Therefore, a fermentative profile with antifungal compounds may be beneficial to avoid aerobic deterioration. Acetic acid has been reported to be an effective compound that improves aerobic stability by reducing yeasts during conservation and by limiting their growth after silage is exposed to air (Kleinschmidt and Kung, 2006; Comino et al., 2014; Ferrero et al., 2019). In the last 20 years, heterofermentative lactic acid bacteria (LAB), and *Lentilactobacillus buchneri* (previously classified as *Lactobacillus buchneri,* Zheng et al., 2020) in particular, have been used as inocula to prevent the aerobic deterioration of silages, because of their ability to produce acetic acid via the anaerobic conversion of moderate amounts of lactic acid to acetic acid and 1,2-propanediol (Oude Elferink et al., 2001; Muck et al., 2018). However, different conditions can influence the activity of LAB and their ability to alter silage quality (Muck et al., 2018), and heterofermentative LAB inoculants have not been consistently effective in improving aerobic stability as a result of the type of forage, the dry matter (DM) of the forage at harvest, variations in the application rate, and the temperature and length of conservation (Kleinschmidt and Kung, 2006; Blajman et al., 2018; Ferrero et al., 2021). Silages with a high DM content (e.g., >38%) are more prone to aerobic spoilage than those with a low content, because the LAB activity is limited by the reduced water activity (a_w) and, consequently, the production of acids is low (Comino et al., 2014). The length of conservation represents a critical point for the quality and stability of silage. A long conservation period in complete anaerobiosis determines a decrease in the microbial count and in particular in the yeast and mold counts, and consequently improves the aerobic stability of silages, regardless the use of silage inocula (Weinberg and Chen, 2013; Ferrero et al., 2019). *L. buchneri*-based inocula often need a long conservation period (>45 to 60 days) to be efficacious (Driehuis et al., 1999). Producers often want to start feeding-out silages from silos after ensiling periods of less than 30 days, thus making it necessary for the acetic acid to be produced earlier by heterofermentative LAB, during ensiling (Ferrero et al., 2019; Nair et al., 2020). *Lentilactobacillus hilgardii* (previously classified as *Lactobacillus hilgardii,* Zheng et al., 2020), was isolated from sugar cane (Ávila et al., 2014) and deposited and patented as silage inocula (European Patent Application EP2826385A1), thereby becoming a possible new inoculum to improve aerobic stability after a short conservation period. Phylogenetic evaluation and fermentation profiling of *L. hilgardii* have revealed that it is closely related to *L. buchneri,* as both possess the ability to degrade lactic acid to acetic acid and to 1,2-propanediol in anaerobic conditions (Heinl et al., 2012; Drouin et al., 2019). Assis et al. (2014) gave the first indication of the use of *L. hilgardii* as inoculum for corn silage. Subsequent studies were conducted to evaluate the impact of *L. hilgardii* on ensiling fermentation and on the aerobic stability of corn silages, and they reported higher concentrations of acetic acid, lower yeast populations, and a higher aerobic stability than uninoculated silages (Reis et al., 2018; Ferrero et al., 2019; Costa et al., 2021).

Since the DM content at harvest and the length of conservation can affect the silage quality and the effectiveness of inocula, the aim of this study has been to evaluate the effects of *L. hilgardii*, the DM content at harvest and the length of conservation on the fermentation quality and aerobic stability of whole crop corn silages.

MATERIALS AND METHODS

Crop and Ensiling

Thirteen dairy farms operating in the western Po plain, northern Italy, were involved in the research. Each farm was asked to grow the same corn hybrid (*Zea mays* L. cultivar P1517W, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) and to manage the crop in almost the same agronomic way. Briefly, seeding date in the first decade of April; theoretical planting density of 75,000 seeds/ha; conventional tillage at 0.25–0.30 m depth; fertilization with 20,000–30,000 kg/ha of liquid slurry (average NPK content of 0.27, 0.24, and 0.31% on fresh basis) applied immediately before plowing, 104 kg/ha of potassium chloride (K, 60%) applied prior to planting, 104 kg/ha of diammonium phosphate (NP, 18 and 46%) applied at planting, and 166 kg/ha of urea (N 46%) top dressed at the 6–8 leaf-stage; pre-emergence weed control with Lumax (mesotrione 37.5 g/l, S-metolachlor 37.5 g/l and terbuthylazine, 12.5 g/l, Syngenta, Basel, Switzerland) at a rate of 3.5 l/ha plus an additional post-emergence application with Titus[§] Mais Extra (nicosulfuron 300 g/kg, rimsulfuron 150 g/kg, DuPont, Milano, Italy) at a rate of 70 g/ha, if necessary; 3–5 irrigations (flooding method, about 200–300 mm each time). In each farm (except one) the crop was harvested at two different maturity stages of growth to obtain different DM contents at ensiling. Corn was harvested, as chopped whole crop, using a precision forage harvester (Claas Jaguar 950, equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cutting length of 12 mm. The DM contents of the forages at harvest ranged from 25 to 45%. The field was divided into blocks, which were subsequently harvested separately to obtain three replicates. The fresh herbage of each plot was divided into two piles (about 50 kg), which were either not treated, as a negative control (CONT), or treated with *Lentilactobacillus hilgardii* CNCM I-4785 (Lallemand SAS, France) at 300,000 cfu/g fresh matter (FM) (LH). The lyophilized microbial inoculant was diluted in sterilized water and applied using a hand sprayer, at a rate of 4 mL/kg of forage, by spraying uniformly onto the forage, which was constantly hand mixed. The same amount of water was added to the CONT treatment. In order to add the targeted amount of LAB, the inoculum was plated on MRS agar (Merck, Whitehouse Station, NY), to which natamycin (0.25 g/L) had been added and incubated as described hereafter. Moreover, an appropriate amount of inoculum was used, on the basis of the measured LAB concentration, to achieve the desired application rate.

The fresh forage was sampled prior to ensiling after the inoculum had been applied. The untreated and treated forages were then ensiled (about 10–14 kg of wet forage) in 20 L plastic silos equipped with a lid that only enabled the release of gas.

The forages were packed by hand in order to reach the same weight of the silo at similar DM contents. Therefore, the final packing densities, on a wet basis, ranged from 669 to 529 kg FM/m^3 depending on DM content. All the laboratory silos were filled within 3 h. The silos were weighed and conserved at ambient temperature in a controlled environment (20 ± 1°C) and were then opened after a conservation period ranging from 13 to 272 days, depending on the experiment. On opening, each silo was weighed again, the first 50 mm layer was discarded, and the remaining silage was mixed thoroughly and sub-sampled to determine the DM content, chemical composition, fermentation profile and the microbial counts. The DM losses due to fermentation were calculated as the difference between the weight of the forage placed in each plastic silo at ensiling and the weight of the silage at the end of conservation, corrected for the DM content of the forage and its respective silage.

After sampling, the silages were subjected to an aerobic stability test, which involved monitoring the temperature increases due to the microbial activity in the samples exposed to air in insulated boxes under a controlled environment. About 3 kg from each silo was allowed to aerobically deteriorate at a controlled temperature (20 ± 1°C) in 17 L polystyrene boxes (290 mm diameter and 260 mm height). A single layer of aluminum foil was placed over each box to prevent drying and dust contamination, but also to allow the air to penetrate. The room and silage temperatures were measured hourly by means of a mini temperature logger (Escort Intelligent Mini, Escort Data Logging Systems Ltd., Auckland, New Zealand). Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased by 2°C above the ambient temperature.

Sample Preparation and Analyses

The pre-ensiled material and the silages were split into five subsamples. One sub-sample was analyzed immediately, for the DM content, by oven drying at 80°C for 24 h. Dry matter was corrected according to Porter and Murray (2001), to consider the volatile compound losses that can take place at 80°C. The second subsample was oven-dried at 65°C to a constant weight and was air equilibrated, weighed and ground in a mill (Cyclotec Tecator, Herndon, VA, United States) to pass a 1 mm screen. The dried samples were analyzed for the total nitrogen (TN), according to the Dumas method (method number 992.23, AOAC International, 2005), using a Primacs SN nitrogen analyzer (Skalar, Breda, The Netherlands), for crude protein (CP) (total N × 6.25) and for ash by ignition (method number 942.05, AOAC International, 2005). The starch concentration was determined according to the AOAC methods (method number 996.11; AOAC International, 2005). Neutral detergent fiber (NDF) was analyzed, using a Raw Fiber Extractor (FIWE, VELP Scientifica, Usmate Velate, Italy), with the addition of heat-stable amylase (A3306, Sigma Chemical Co., St. Louis, MO) and expressed on a DM basis, including residual ash, as described by Van Soest et al. (1991). Acid detergent fiber (ADF) was analyzed and expressed on a DM basis, including residual ash (Robertson and Van Soest, 1981). A third fresh sub-sample was used to determinate the water activity (a_w), pH, nitrate (NO_3) and the buffering capacity. a_w was measured at 25°C, on a fresh sample, using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled mirror dew point technique. The fresh forage was extracted for pH and nitrate using a Stomacher blender (Seward Ltd., Worthing, United Kingdom), for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate concentration was determined in the water extract, through semi-quantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3/kg DM). The pH was determined using a specific electrode (DL21 Titrator, Mettler Toledo, Milan, IT). The buffering capacity was determined in the water extract, as described by Playne and McDonald (1966). A fourth sub-sample was extracted, using a Stomacher blender, for 4 min in H_2SO_4 0.05 mol/L at a 4:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 mL of silage acid extract was filtered with a 0.20-μm syringe filter and used for quantification of the fermentation products. The lactic and monocarboxylic acids (acetic, propionic and butyric acids) were determined, by means of high-performance liquid chromatography (HPLC), in the acid extract (Canale et al., 1984). Ethanol and 1,2-propanediol were determined, by means of HPLC, coupled to a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). The fifth subsample was used for the microbial analyses.

In order to conduct the microbial counts, an aliquot of 30 g was transferred into a sterile homogenization bag, suspended 1:9 w/v in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per liter) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd., London, United Kingdom). Serial dilutions were prepared, and the yeast and mold numbers were determined using the pour plate technique by inoculating, in duplicate, 1 mL of dilution on Yeast Extract Glucose Chloramphenicol Agar (YGC agar, DIFCO, West Molesey, Surrey, United Kingdom), after incubation at 25°C for 3 and 5 days for yeast and mold, respectively. The yeast and mold colony forming units (cfu) were enumerated separately, according to their macromorphological features, on plates that yielded 15–150 cfu. Since lot of samples had very low yeast and mold counts, when the plates of the original dilution yielded fewer than 15 colonies, actual plate count was recorded and reported as log transformed value. A detection limit of 1.00 log cfu/g (10 cfu/g of silage) has been assumed when plates from original dilution yielded no colony forming unit. The LAB counts were determined on MRS agar, to which natamycin (0.25 g/L) had been added, by incubating the Petri plates at 30°C for 3 days in anaerobic jars with a gas generating system [AnaeroGenTM, Thermo Fisher Scientific, Rodano (MI), Italy]. Since LAB are facultative anaerobe bacteria, anaerobic incubation was chosen to improve the selectivity of the media against *Bacillus* spp. (Spoelstra et al., 1988). The LAB colony forming units were enumerated on plates that yielded 30–300 cfu.

Statistical Analysis

Data (n = 150) pertaining to the inoculum application (CONT, n = 75; LH, n = 75), the DM content and time of conservation were compared. The data were divided into three classes of DM content, that is, low DM (<33% DM, n = 43), medium DM

(33–38% DM, n = 58) and high DM (>38% DM, n = 49). Moreover, the data were divided into four classes of conservation times, that is, very short (<15 days, n = 36), short (15–30 days, n = 42), medium (31–120 days, n = 42) and long (>120 days, n = 30). The nitrate content was corrected for the dilution factor and expressed on a DM basis. The microbial counts were $\log_{10}$ transformed and presented on a wet weight basis. The values below the detection limit for yeast and mold (detection level: 10 cfu/g of silage) were assigned a value corresponding to half of the detection limit to calculate the average value. An unpaired t-test was used to analyze the effect of inoculum application on the fermentative characteristics, microbial counts, chemical characteristics, and aerobic stability. One-way analysis of variance was used to analyze the effect of the conservation time and DM content on the fermentative characteristics, microbial counts, chemical characteristics, and aerobic stability. The data were analyzed using the General Linear Model of the Statistical Package for Social Science (v 26.0, SPSS Inc., Chicago, Illinois, the United States). When the calculated values of F were significant, the Tukey *post hoc* test (P < 0.05) was used to interpret any significant differences among the mean values. Multiple regression analysis was used to correlate the acetic acid concentration, yeast count and aerobic stability values with the days of conservation (DAY), silage DM concentration, and treatment (inoculation with LH), using the Draper and Smith (1998) stepwise selection procedure to select the best regression model at a 0.05 probability level. All the determination coefficients (r^2 or R^2) reported in this paper were adjusted for degrees of freedom. The data were analyzed across experiments for regression analysis using SPSS v. 26.

RESULTS

The chemical and microbial characteristics of the corn at ensiling are reported in **Table 1**. The DM content of the silages ranged from 25.2 to 45.1%. The pH was typical of corn at harvesting, with a mean value of 5.72. a_w and the buffering capacity ranged from 0.981 to 0.999 and from 23 to 69 meq/kg DM, respectively. The yeast, mold and LAB counts were on average 6.72, 5.40, and 7.76 log cfu/g, respectively. The nutritional composition of the silages showed a high variability, with starch and NDF ranging from 22 to 37% DM, and from 32 to 49% DM, respectively.

TABLE 1 | Chemical and microbial characteristics of corn silages at ensiling.

Items	Mean	*SD*	Min	Max
DM (%)	34.7	5.81	25.2	45.1
pH	5.72	0.34	5.54	6.03
NO_3 (mg/kg DM)	<100	61	<100	156
a_w	0.992	0.006	0.981	0.999
Buffering capacity (meq/kg DM)	44	15	23	69
Yeast (log cfu/g)	6.72	0.27	6.03	7.37
Mold (log cfu/g)	5.40	0.48	4.54	6.65
Lactic acid bacteria (log cfu/g)	7.76	0.76	5.63	8.74
Crude protein (% DM)	7.1	0.86	5.8	8.3
Ash (% DM)	4.5	0.90	2.8	5.7
Starch (% DM)	30.5	4.20	21.7	37.2
NDF (% DM)	40.2	4.20	32.0	48.8
ADF (% DM)	20.3	2.96	14.9	25.7
Hemicellulose (% DM)	19.9	1.38	17.0	23.2
ADL (% DM)	4.1	0.49	2.9	5.0

ADF, acid detergent fiber; ADL, lignin; a_w, activity water; cfu, colony forming unit; DM, dry matter; NDF, neutral detergent fiber; SD, standard deviation.

The Pearson correlation coefficients of the chemical, fermentative and microbial characteristics of the corn silages are reported in **Table 2**. The length of conservation was positively correlated with the pH, lactic and acetic acids, 1,2-propanediol, and DM losses. On the other hand, it was negatively correlated with the microbial counts (yeast, mold, and lactic acid bacteria) and fiber component of the silages (NDF, ADF, and hemicellulose). Negative correlations were detected between the DM content and lactic and acetic acids, aerobic stability, and nutritional parameters, except for starch. The DM content was positively correlated with the yeast and mold counts, and starch content. The pH was strongly negatively correlated with the lactic acid content and positively with the LAB count. Acetic acid was negatively correlated with the yeast and mold counts. The DM losses were found to be correlated positively to the days of conservation and to the DM content.

The chemical, fermentative and microbial characteristics of the corn silages, untreated or treated with *L. hilgardii,* are reported in **Table 3**. The application of inoculum determined higher amounts of acetic acid (P = 0.013) and a lower lactic-to-acetic ratio than CONT. 1,2-propanediol was higher in LH than in CONT (P < 0.001). The yeast count was lower (P = 0.021) and aerobic stability was higher (P = 0.044) in LH than in CONT. The DM losses during fermentation and the nutritional parameters were not affected by the application of inoculum. All the other nutritional characteristics of the silages resulted to be unaffected by the LH inoculum.

The chemical, fermentative and microbial characteristics of the corn silages for the three classes of DM content are reported in **Table 4**. The DM content influenced the concentration of fermentative products to a great extent, with a decrease in the lactic and acetic acids from low to high DM silages (P < 0.001), even though the pH remained almost constant. On the other hand, the ethanol content increased from low to high DM silages (P < 0.001). The yeast count was higher in the high DM silages than in the low DM ones (4.15 vs. 2.17 log cfu/g), and the aerobic stability was higher in the low DM silages than in the medium and high DM ones. The LAB counts decreased slightly as the DM content increased. The high DM silages showed lower (P < 0.001) NDF, ADF, hemicellulose and crude protein contents and a higher starch content (P < 0.001) than the low DM ones.

The chemical, fermentative and microbial characteristics of the corn silages for the four classes of days of conservation are reported in **Table 5**. The pH decreased as the length of conservation increased (P < 0.001). The lactic and acetic acid contents were higher for the Long conservation period than for the shorter period, whereas the lactic-to-acetic ratio decreased as the conservation period increased. 1,2-propanediol was higher than 1.0 g/kg DM after 30 days of conservation. The yeast and LAB counts decreased as the conservation period

TABLE 2 | Pearson correlation coefficients of chemical and microbiological characteristics of corn silages (*n* = 150).

	DAY	DM	pH	LA	AA	ET	1,2-PD	Yeast	Mold	LAB	AS	NDF	ADF	HEM	ASH	CP	STARCH
DM	−0.122																
pH	−0.540***	0.143															
LA	0.654***	−0.751***	−0.418***														
AA	0.718***	−0.573***	−0.393***	0.771***													
ET	−0.073	0.220*	0.212*	−0.122	−0.116												
1,2-PD	0.439***	0.021	−0.280**	0.128	0.592***	−0.013											
Yeast	−0.691***	0.551***	0.423***	−0.783***	−0.801***	0.139	−0.410***										
Mold	−0.296**	0.339***	0.186*	−0.392***	−0.388***	0.082	−0.156	0.392***									
LAB	−0.687***	−0.093	0.515***	−0.412***	−0.337***	−0.000	−0.100	0.342***	0.134								
AS	0.600***	−0.345***	−0.254*	0.624***	0.598***	−0.063	0.293**	−0.737***	−0.269**	−0.398***							
NDF	−0.331***	−0.707***	0.101	0.272*	0.218*	−0.050	−0.123	−0.055	−0.185*	0.348***	−0.133						
ADF	−0.214*	−0.764***	−0.018	0.383***	0.291**	−0.020	−0.0915	−0.1565	−0.158	0.262*	0.008	0.953***					
HEM	−0.431***	−0.545***	0.234*	0.100	0.100	−0.082	−0.146	0.076	−0.194*	0.410***	−0.291**	0.927***	0.771***				
ASH	0.127	−0.745***	−0.108	0.569***	0.598***	−0.293**	0.124	−0.473***	−0.368***	−0.019	0.141	0.690***	0.602***	0.709***			
CP	0.288**	−0.575***	−0.266*	0.558***	0.634***	−0.374***	0.231*	−0.477***	−0.369***	−0.188*	0.178*	0.525***	0.455***	0.543***	0.888***		
STARCH	0.091	0.844***	0.167	−0.512***	−0.406***	0.195*	0.060	0.321***	0.219*	−0.154	−0.139	−0.862***	−0.923***	−0.675***	−0.717***	−0.591***	
LOSSES	0.470***	0.403***	−0.347***	0.027	0.171	0.374***	0.400***	−0.153	0.122	−0.401***	0.242*	−0.478***	−0.319***	−0.612***	−0.445***	−0.291**	0.325***

AA, acetic acid; ADF, acid detergent fiber; AS, aerobic stability; DAY, days of conservation; DM, dry matter; ET, ethanol; HEM, hemicellulose; LA, lactic acid; LAB, lactic acid bacteria; NDF, neutral detergent fiber; CP, crude protein; 1,2-PD, 1,2-propanediol.
**P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.*

TABLE 3 | Chemical, fermentative and microbiological characteristics of corn silages as affected by inoculum.

Items	CONT	LH	SEM	*P*-value
	***n* = 75**	***n* = 75**		
DM (%)	36.4	36.2	0.438	0.718
pH	3.76	3.77	0.006	0.297
Lactic acid (g/kg DM)	49.7	49.6	1.070	0.991
Acetic acid (g/kg DM)	11.9	13.7	0.377	0.013
Lactic-to-acetic ratio	4.3	3.8	0.060	<0.001
Ethanol (g/kg DM)	11.8	12.3	0.313	0.413
1,2-Propanediol (g/kg DM)	0.1	1.3	0.127	<0.001
Yeast (log cfu/g)	3.35	2.83	0.114	0.021
Mold (log cfu/g)	<1.00	<1.00	–	–
LAB (log cfu/g)	7.43	7.88	0.075	0.002
Aerobic stability (h)	96	116	5.005	0.044
DM losses (%)	2.21	2.35	0.047	0.129
Crude protein (% DM)	7.5	7.6	0.082	0.797
Ash (% DM)	4.6	4.8	0.096	0.290
Starch (% DM)	34.0	33.7	0.327	0.729
NDF (% DM)	38.0	38.7	0.361	0.318
ADF (% DM)	19.8	20.2	0.212	0.367
Hemicellulose (% DM)	18.2	18.5	0.171	0.324

ADF, acid detergent fiber; cfu, colony forming unit; DM, dry matter; LAB, lactic acid bacteria; NDF, neutral detergent fiber; SEM, standard error of the mean.

TABLE 4 | Chemical, fermentative and microbiological characteristics of corn silages as affected by DM content.

Items	Low DM <33%	Medium DM 33–38%	High DM >38%	SEM	*P*-value
	***n* = 43**	***n* = 58**	***n* = 49**		
DM (%)	29.4^{c}	36.2^{b}	42.4^{a}	0.438	<0.001
pH	3.75	3.77	3.76	0.006	0.223
Lactic acid (g/kg DM)	61.5^{a}	49.1^{b}	39.9^{c}	1.070	<0.001
Acetic acid (g/kg DM)	15.8^{a}	13.6^{b}	9.2^{c}	0.377	<0.001
Lactic-to-acetic ratio	4.0^{b}	3.8^{b}	4.5^{a}	0.060	<0.001
Ethanol (g/kg DM)	10.9^{b}	10.7^{b}	14.8^{a}	0.313	<0.001
1,2-Propanediol (g/kg DM)	0.57	0.95	0.54	0.127	0.316
Yeast (log cfu/g)	2.17^{c}	2.88^{b}	4.15^{a}	0.114	<0.001
Mold (log cfu/g)	<1.00	<1.00	1.39	–	–
LAB (log cfu/g)	7.94^{a}	7.66^{ab}	7.39^{b}	0.075	0.018
Aerobic stability (h)	141^{a}	97^{b}	85^{b}	5.005	<0.001
DM losses (%)	2.09^{b}	2.09^{b}	2.68^{a}	0.047	<0.001
Crude protein (% DM)	8.0^{a}	8.0^{a}	6.2^{b}	0.082	<0.001
Ash (% DM)	5.3^{a}	5.1^{a}	2.9^{b}	0.096	<0.001
Starch (% DM)	29.9^{c}	34.6^{b}	37.8^{a}	0.327	<0.001
NDF (% DM)	41.6^{a}	38.3^{b}	33.9^{c}	0.361	<0.001
ADF (% DM)	22.4^{a}	19.4^{b}	17.8^{c}	0.212	<0.001
Hemicellulose (% DM)	19.3^{a}	18.9^{a}	16.1^{b}	0.171	<0.001

Means within a row with different superscripts differ (P < 0.05).
ADF, acid detergent fiber; cfu, colony forming unit; DM, dry matter; LAB, lactic acid bacteria; NDF, neutral detergent fiber; SEM, standard error of the mean.

increased, and the aerobic stability increased from 62 to 159 h. The DM losses increased as the conservation period increased, and indirectly influenced the increase in the crude protein,

TABLE 5 | Chemical, fermentative and microbiological characteristics of corn silages as affected by time of conservation.

Items	Very short <15 days	Short 15–30 days	Medium 31–120 days	Long >120 days	SEM	*P*-value
	n = 36	*n* = 42	*n* = 42	*n* = 30		
DM (%)	35.6	37.2	36.6	35.3	0.438	0.419
pH	3.82[a]	3.78[b]	3.73[c]	3.71[c]	0.006	<0.001
Lactic acid (g/kg DM)	41.2[c]	44.5[c]	51.8[b]	63.9[a]	1.070	<0.001
Acetic acid (g/kg DM)	9.4[c]	10.7[c]	13.9[b]	18.0[a]	0.377	<0.001
Lactic-to-acetic ratio	4.5[a]	4.3[a]	3.8[b]	3.7[b]	0.060	<0.001
Ethanol (g/kg DM)	11.0[b]	11.8[ab]	12.1[ab]	13.8[a]	0.313	0.030
1,2-Propanediol (g/kg DM)	0.11[c]	0.23[bc]	1.04[ab]	1.73[a]	0.127	<0.001
Yeast (log cfu/g)	4.08[a]	3.44[ab]	2.90[b]	1.68[c]	0.114	<0.001
Mold (log cfu/g)	1.45	1.09	<1.00	<1.00	–	–
LAB (log cfu/g)	8.41[a]	8.19[a]	7.16[b]	6.72[c]	0.075	<0.001
Aerobic stability (h)	62[a]	87[a]	125[b]	159[c]	5.005	<0.001
DM losses (%)	1.95[c]	2.05[c]	2.41[b]	2.84[a]	0.047	<0.001
Crude protein (% DM)	7.2[b]	7.5[b]	7.6[ab]	8.1[a]	0.082	0.025
Ash (% DM)	4.5	4.6	4.7	5.0	0.096	0.389
Starch (% DM)	33.9	33.5	33.9	34.1	0.327	0.906
NDF (% DM)	39.9[a]	38.6[ab]	38.2[ab]	36.2[b]	0.361	0.006
ADF (% DM)	20.7	19.9	19.9	19.3	0.212	0.176
Hemicellulose (% DM)	19.3[a]	18.7[a]	18.2[a]	16.9[b]	0.171	<0.001

Means within a row with different superscripts differ (P < 0.05).
ADF, acid detergent fiber; cfu, colony forming unit; DM, dry matter; LAB, lactic acid bacteria; NDF, neutral detergent fiber; SEM, standard error of the mean.

TABLE 6 | Regression models of acetic acid, yeast count, and aerobic stability on days of conservation, dry matter content and treatment with *L. hilgardii* as independent variables.

	Model	RMSE	Adjusted R^2	*P*-value
Acetic acid (g/kg DM)	$y = 16.81 + 0.06012 \times DAY - 0.0071 \times DM^2 + 1.731 \times TREAT$	2.074	0.798	<0.001
Yeast (log cfu/g)	$y = 1.478 - 0.0084 \times DAY + 0.0023 \times DM^2 - 0.4812 \times TREAT$	0.775	0.691	<0.001
Aerobic stability (h)	$y = 202.3 + 0.3658 \times DAY - 3.956 \times DM + 18.85 \times TREAT$	44.9	0.462	<0.001

DAY, days of conservation; DM, dry matter content (%); TREAT, treatment with L. hilgardii (0 = not treated; 1 = treated); RMSE, root mean square error.

whereas hemicelluloses decreased and determined a reduction in the NDF content.

The acetic acid, yeast count and aerobic stability regression models, are reported in **Table 6** with the days of conservation (DAY), DM content (linear or quadratic, DM or DM^2) and treatment with *L. hilgardii* (0 = untreated; 1 = treated with LH) as the independent variables. The acetic acid content and yeast count of the silages were influenced by the days of conservation, DM^2 and treatment, with adjusted coefficients of determination of 0.798 and 0.691, respectively. The application of the LH inoculum determined an increase in the acetic acid (1.73 g/kg DM) and a reduction of the yeast count of 0.48 log cfu/g. The days of conservation influenced the aerobic stability (about 3.7 h for each further 10 days of conservation) and the treatment with LH on average increased for 18.9 h of aerobic stability. On the other hand, the DM content of the silage determined a reduction of 39.6 h every 10% units of increase of DM.

The acetic acid content, yeast count and aerobic stability, as affected by the treatment with *L. hilgardii*, the DM content and the days of conservation are shown in **Figures 1–3**, respectively. Acetic acid was influenced by the LH treatment and shows an interaction with the DM and DAY of conservation. The yeast count decreased during conservation, with lower values in low DM silages than in high DM silages, while the silages inoculated with LH presented lower values than C ($P < 0.001$). Aerobic stability was higher in the treated silages than in the untreated ones, higher in the low DM silages than the high DM ones, and higher in the long conservation periods than the short ones ($P < 0.001$).

DISCUSSION

The present study indicated that the aerobic stability of silage is closely related to the yeast count and, albeit indirectly, to the antifungal activity of acetic acid, and that several interacting factors can influence its magnitude. Heterofermentative LAB inocula have been developed to reduce the yeast count over conservation and to slow down or to inhibit their activity after silo opening, thus improving the aerobic stability of silages (Muck et al., 2018). LAB strains selection is based on their ability to fast growth and to compete with epiphytic microorganisms especially under sub-optimal conditions (e.g., high DM content or low temperature). However, a notable change in LAB composition have been detected during the first fermentation phase (Drouin et al., 2019), and many of the inocula strains are not fast in dominating the fermentation. Under farm conditions, corn is often harvested when the plants are lower or higher in DM than the optimal concentration (from 30 to 38% DM) due to an inadequate capacity to harvest large amounts of forage over a short period of time (Windle et al., 2014). Furthermore, several farmers choose to harvest corn at high concentrations of DM to obtain higher yields and starch concentrations. However, this leads to greater packing difficulties and the resulting silage often spoils rapidly when exposed to air (Comino et al., 2014; Windle et al., 2014). A high DM content, because of a reduced a_w, limits the LAB activity, with a consequent reduction in volatile fatty acid production (Comino et al., 2014), and in particular of acetic acid, which is known to strongly contrast yeast activity in the presence of air. In our experiment, a restriction of fermentation was observed from wetter to drier silages, with the latter having lower acetic acid concentrations than 10 g/kg DM. This finding is in agreement with the results of Der Bedrosian et al. (2012), who reported that the concentration of acetic acid was higher (14.2 g/kg DM) in 32% DM than in 41% DM (11.6 g/kg DM) corn silage, and of those of Comino et al. (2014), who found a progressive restriction of acetic acid production in corn silage of

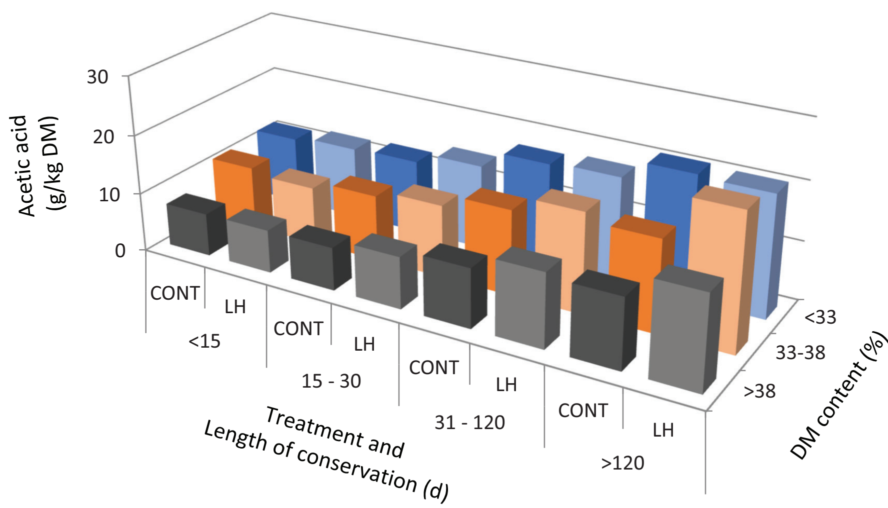

FIGURE 1 | The acetic acid content as affected by treatment with *Lentilactobacillus hilgardii*, DM content and days of conservation.

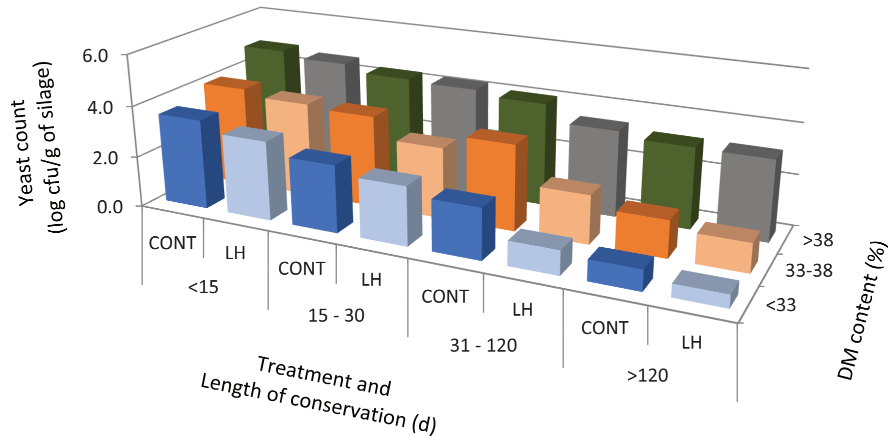

FIGURE 2 | The yeast count as affected by treatment with *Lentilactobacillus hilgardii*, DM content and days of conservation.

more than 20 g/kg DM when the DM content increased from 26 to 43%. Lower concentrations of acetic acid in high DM corn silages are considered to be one of the factors that contribute the most to explaining why these silages tend to be less aerobically stable than low DM corn silages (Der Bedrosian et al., 2012). In the present experiment, the lower amount of acetic acid in silages with a higher DM content than 38% resulted in a higher yeast count than 4 log cfu/g. Therefore, in these silages, the aerobic stability was lower than the aerobic stability observed for wetter silages.

Aerobic stability can also be influenced to a great extent by the conservation time. A long conservation period in complete anaerobiosis has been reported to determine a decrease in the yeast count (Weinberg and Chen, 2013; Ferrero et al., 2019). Weinberg and Chen (2013) analyzed the fermentation profile and microbial count of corn silages opened after 1 week to 12 months of conservation. They found an increase in acetic acid of 8–47 g/kg DM, which resulted in a decrease in the yeast count and an improvement in aerobic stability. The same trend pertaining to an acetic acid increase, yeast count decrease and aerobic stability increase was reported by Kleinschmidt and Kung (2006), who investigated untreated corn ensiled at 37% DM, over an ensiling period of 14–361 days. Furthermore, Der Bedrosian et al. (2012) reported that the acetic acid content increased steadily in untreated corn silage with the length of storage, from 9.8 g/kg DM after 45 days of ensiling to 17.1 g/kg DM after 360 days of ensiling. In agreement with the paper mentioned above (Kleinschmidt and Kung, 2006; Der Bedrosian et al., 2012), a long conservation period in the current experiment resulted in an increase in acetic acid content, a reduction in the yeast count and an improvement in aerobic stability. These effects were more evident in the wetter silages than in the drier ones, thus highlighting a positive synergistic effect between the DM of silage and the length of the conservation period. The wetter silages

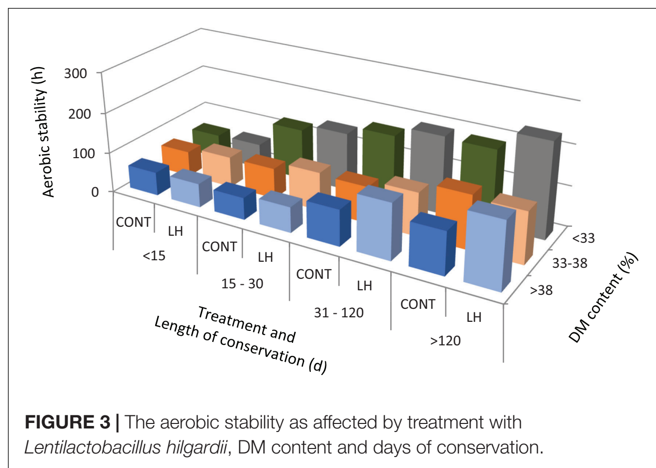

FIGURE 3 | The aerobic stability as affected by treatment with *Lentilactobacillus hilgardii*, DM content and days of conservation.

therefore had a lower yeast count and higher aerobic stability than the drier silages for a given length of conservation. This is in agreement with the results of Ferrero et al. (2021) who observed, on corn silages ensiled at 42% DM from 15 to 100 days, that the reduction in the yeast count and the increase in aerobic stability during conservation was less marked than in another experiments (Ferrero et al., 2019) in which corn silages were ensiled, for the same conservation periods but at lower DM contents (36.3 and 34.0%).

Several authors have reported that the heterofermentative LAB *L. buchneri* (the most frequently used heterofermentative LAB inoculum) does not show a consistent effect on corn silages after shorter periods of conservation than 45–60 days (Driehuis et al., 1999; Kleinschmidt and Kung, 2006; Ferrero et al., 2019) or on silages with a higher DM than 38% (Hu et al., 2009; Comino et al., 2014; Xu et al., 2019). To overcome this drawback, recent research has been directed toward finding heterofermentative *Lactobacillus* strains which are able to work rapidly after silo closure. A strain of *L. hilgardii* was isolated from sugarcane silage in Brazil (Ávila et al., 2014) and was produced and industrialized to be active after a short conservation period. Reis et al. (2018) found an increase in acetic acid content after 19 days of ensiling corn silage, but these authors did not perform an aerobic stability test. The same authors however found an increase in aerobic stability in silages treated with LH after 103 days of conservation (Reis et al., 2018). In the study of Ferrero et al. (2019), the addition of LH alone increased the aerobic stability in one out of two corn silage trials, with higher aerobic stabilities observed after 15 and 30 days of conservation. Drouin et al. (2019) found a higher acetic acid production in silages treated with LH than in uninoculated ones already after the first day of ensiling. Costa et al. (2021) found that silages treated with *L. hilgardii* had higher aerobic stability, and lower numbers of yeasts than untreated ones. Ferrero et al. (2021) did not find any differences after 15 or 30 days of conservation between treated and untreated silages, probably because of the DM content of the silages was higher than 40%. In our experiment, *L. hilgardii* was able to dominate the fermentation and to modify the fermentation profile, with a high production of acetic acid and 1,2-propanediol, which is the typical end-product of *L. buchneri* group bacteria (Oude Elferink et al., 2001; Muck et al., 2018). This fermentative profile resulted in a reduction in the yeast count and an average increase in aerobic stability of 20 h, compared to the untreated silages.

In the present paper, we performed a regression model with the days of conservation, the DM content and inoculation with *L. hilgardii* to correlate the acetic acid content, yeast count and aerobic stability. Acetic acid has been found to increase as the conservation period increases and to decrease as the DM content increases. This can be explained by the fact that heterolactic bacteria act after homolactic ones, and their activity is less marked in high DM silages (Pahlow et al., 2003). The inoculation with LH determined an increase in the acetic acid (+1.73 g/kg DM). As expected, the yeast count was reduced as the time of conservation increased in a DM dependent manner, and the inoculation with LH determined an average reduction of the count of 0.48 log cfu/g. The aerobic stability increased by about 3.7 h for every 10 days of conservation, and on average by 18.9 h for the LH treatment, whereas it decreased for an increasing DM content of the silages, as already discussed above, and as reported by several authors (Comino et al., 2014; Ferrero et al., 2021).

The treatment with *L. hilgardii* did not show any differences in the nutritional composition of the silages. On the other hand, the DM content influenced the composition of the corn silage to a great extent. As expected, the increase in the DM content of silage determined an increase in the starch content and a reduction in the NDF content. This can be useful for animal nutrition as starch represents a source of energy for dairy and beef cattle. However, no changes were observed during conservation, although it has been reported that starch degradability increases during storage in corn silages with a high DM content, and that starch degradability increases during storage (Hoffman et al., 2011; Gerlach et al., 2018).

The results of this experiment suggest that higher aerobic stability could be achieved in corn silages by ensiling at medium or low DM contents, or by increasing the length of conservation if a higher DM content at ensiling is needed. The results also suggest that inoculation with *L. hilgardii* helps to improve the aerobic stability of corn silages by enhancing the production of acetic acid, which determines a greater reduction in the yeast counts than those observed in uninoculated silages. The best results have been found in medium DM content silages (i.e., 33–38% DM) and for microbiological stabilized silages (after a medium to long conservation period in complete anaerobiosis, i.e., >30 days). Further study will be needed to evaluate the effect of DM content, length of conservation and treatment with *L. hilgardii* under farm conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FF, ET, and GB conceived and designed the experiments. ET and FF analyzed the data. FF wrote the original draft of the

manuscript. ET and GB wrote, reviewed, and edited the maunscript. GB supervised, administered the project, and acquired funding. All authors performed the experiments.

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Effects of Lactic Acid Bacteria on Microbial Metabolic Functions of Paper Mulberry Silage: A BIOLOG ECO Microplates Approach

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Lactic acid bacteria occupy an important position in silage microorganisms, and the effects of exogenous lactic acid bacteria on silage quality have been widely studied. Microbial metabolism has been proved as an indicator of substrate utilization by microorganisms. Paper mulberry is rich in free carbohydrate, amino acids, and other components, with the potential to be decomposed and utilized. In this study, changes in the microbial metabolism characteristics of paper mulberry silage with *Lactiplantibacillus plantarum* (LP) and *Lentilactobacillus buchneri* (LB) were studied along with a control (CK) using BIOLOG ECO microplates. The results showed that average well-color development (AWCD), Shannon diversity, Shannon evenness, and Simpson diversity exhibited significant temporal trends. LB and LP responded differently in the early ensiling phase, and the AWCD of LB was higher than LP at 7 days. Principal component analysis revealed that CK, LB, and LP samples initially clustered at 3 days and then moved into another similar cluster after 15 days. Overall, the microplates methodology applied in this study offers important advantages, not least in terms of accuracy.

Keywords: paper mulberry, silage, lactic acid bacteria, additives, BIOLOG ECO, microbial communities, metabolic function

INTRODUCTION

Paper mulberry (*Broussonetia papyrifera* L.) is rich in crude protein and widely adaptable in temperate, subtropical, and tropical regions. It has been recently developed as a new type of fodder (Bingwen et al., 2019; Du et al., 2020). As an important technique for preserving forage nutrients in the context of seasonal harvests in climates characterized by high temperatures and plentiful precipitation, ensiling has been proved to be an easy way to preserve paper mulberry (Zhang et al., 2019). In the process of ensiling, chemical components are either maintained or partially converted into distinct substances by microorganisms. In addition to carbohydrates, paper mulberry also contains abundant amino acids (Wang et al., 2019) and many other bioactive compounds (Ming et al., 2011; Cao et al., 2020). At the beginning of silage fermentation, undesirable microbial compositions containing yeast, mold, and enteric bacteria are commonly detected, which

can directly and indirectly affect silage safety (Muck, 2010). Exogenous lactic acid bacteria are often employed to accelerate the procedure, inhibit the growth of undesirable microorganisms, and improve the quality of silage. However, little is known about the metabolic characteristics of microbial communities in paper mulberry silage, which significantly limits the optimization of the processing technology of silage fermentation.

The plate colony counting method (Kramer et al., 1979), nucleic acid amplification technique (Zhao and Dong, 2012), and cell morphology (Smith et al., 2016) are conventional approaches for evaluating the structure and composition of microbial communities. However, considering the complicated operation and assays and low repeatability, the overall status of microbial communities is difficult to describe quickly, conveniently, and comprehensively by these above methods. Thus, it is highly desirable to develop a new method for assessing the status of microbial communities. The BIOLOG ECO microplate is a technique for characterizing microbial communities derived from samples based on metabolic functions. Profiles of metabolic functions are assessed using a redox system with 31 different sole carbon sources and one blank control (water), repeated three times (96 wells in total) in each microplate (Gryta et al., 2014). In these plastic microtiter plates, the grade of substrate utilization by microorganisms is quantified based on the absorbance of each well. Different carbon sources, which represent particular metabolic functions, can be used to compare microbial communities in different samples, indicating that the BIOLOG ECO microplate methodology has the potential to measure and analyze the status of microbial communities (Choi and Dobbs, 1999).

Due to the biological and biochemical properties of BIOLOG ECO microplate, it is a relatively simple and quick method for describing the ecological diversity and community-level status of environmental microorganisms. Indeed, it has been widely used in various contexts, such as soils (Bradley et al., 2006), fertilizer (Wei et al., 2018), stored rice (Ge et al., 2018), sludge (Wang et al., 2018), and wastewater (Choi and Dobbs, 1999). However, its potential in the field of silage remains to be elucidated.

In view of the foregoing, this study aimed to investigate the metabolic functions in paper mulberry silage inoculated with *Lactiplantibacillus plantarum* (LP) and *Lentilactobacillus buchneri* (LB), based on an analysis of metabolic profiles, activity, and diversity. The BIOLOG ECO microplate technique was utilized to assess the effect of silage duration (from 0 to 60 days) on the characteristics of different microbial communities. The results of this study provide insights into the effects of ensiling and lactic acid bacteria additives on the metabolic function of microbial communities over time.

MATERIALS AND METHODS

Silage and Samples Preparation

The LP was from woody forage silages while LB was from Sichuan pickles (Gaofuji, China). After isolation and purification, both strains were transformed to additives via lyophilization according to a previous study (Zhang et al., 2016). Paper mulberry was harvested at 120 cm and chopped into approximately 2 cm long pieces using a combine harvester. Fragments were separately exposed to different ensiling treatments with an equal amount of distilled water and divided into three groups, namely additives of LP, additives of LB, and the blank control (CK). The additives were given at a level of 10^6 cfu per gram of raw material (RM) and mixed evenly. Every 3 kg of forage was compressed to a density of around 593 kg/m^3 and sealed in a polyethylene bag (45 cm × 75 cm) by a vacuum sealing machine. For each treatment group, four replicates (one as backup) were prepared on each ensiling day. A total of 60 bags was stored in a dark room at room temperature (around 25°C). Silages collected at 0 day (raw material), 3, 7, 15, 30, and 60 days were applied to observe the changes in microbial metabolism. Forages were homogenized and sub-samples of about 100 g were separated by coning and quartering methods repeatedly. Next, 10 g was removed and dipped in 90 mL of sterilized saline solution (0.85% NaCl). The mixture was vortex-homogenized for 30 s and 1 h at 4°C, 150 rpm by a Controlled Crystal Oscillator (RONGHUA, China). To enrich the microorganisms, filtration was undertaken through a double-layer sterile gauze and centrifuged at 7,800 rpm for 15 min at 4°C. The pellet was resuspended and washed with the above solution twice, and finally made up to 100 ml. Each aliquot of well-mixed diluent (150 μL) was added into the wells of BIOLOG ECO microplates completely within 1 h. Once inoculated, the microplates were placed in a dark and anaerobic container at 25°C. During incubation, the absorbance values were recorded at a wavelength of 590 nm at an interval of 24 h for a total of 240 h using a microplate readers (TECAN, Swiss).

BIOLOG ECO Analysis

The production of NADH via microbial respiration reduces tetrazolium dye to formazan, resulting in chroma development expressed as Average Well-Color Development (AWCD) (Garland and Mills, 1991). The wells of BIOLOG ECO microplates were filled with six carbon sources, namely carboxylic acids, carbohydrates, amino acids, polymers, and miscellaneous and amines/amides, which were used to measure the capability of microorganisms with different carbon sources in microbial communities (Ge et al., 2018). To detect the changes, it was necessary to study the differences between their metabolic characteristics with different carbon sources. Evaluation was conducted by principal component analysis (PCA) with transformed data (Kong et al., 2013). Absorbance value data were calculated as AWCD and standardized as R_{si} via the following equations:

$$\mathrm{AWCD} = \sum_{i=1}^{31} (C_i - R) \big/ 31 \quad (1)$$

$$R_{si} = (C_i - R) \big/ \mathrm{AWCD} \quad (2)$$

In each replicate, C_i represents the absorbance value of each reaction well at 590 nm, and R represents the absorbance value of the water blank well. Data of (C_i – R) less than 0.06 were regarded as zero (Classen et al., 2003). Data from the 144 h were used for the PCA.

Microbial Diversity Analysis

The Shannon-Wiener diversity index (H'), Shannon evenness index (E), and Simpson diversity index (D) were used to investigate metabolic functional diversity at the community level (Hiraishi et al., 1991; Hu et al., 1999; Keylock, 2005).

$$P_i = (C_I - R) \Big/ \sum (C_i - R) \quad (3)$$

$$H^{'} = -\sum P_i \ln P_i \quad (4)$$

$$E = H^{'} \Big/ \ln S \quad (5)$$

$$D = 1 - \sum P_i^2 \quad (6)$$

where P_i represents the ratio of the absorbance value in the i-th (1–31) well to the total absorbance value of all wells. S represents the number of utilized carbon sources among 31 carbon sources.

Substrate Utilization Abundance

To visualize the utilization of various substrates in the BIOLOG ECO microplates by the microorganisms as the average absorbency proportion of each carbon source, the data were divided into six groups according to the type of carbon source based on the following equations:

$$f_i = \frac{C_i}{\sum_{i=1}^{31} C_i} \quad (7)$$

$$F_i = \frac{1}{n} \sum_{i=1}^{n_j} f_i \quad (8)$$

where f_i represents the OD fraction of carbon source species i, F_j represents the average OD fraction of the j kind of carbon source, and nj represents the number of wells in the replicate.

Statistical Analysis

Using the JMP 14 software package (SAS Institute), the data were processed statistically and submitted to the PCA. Tukey's test was used for multiple comparisons, with statistically significant differences at $p < 0.05$. All figures were generated with Origin 9.4.

RESULTS

Microbial Metabolic Activity

To evaluate the metabolic activity of microbial communities of silages and raw materials, the development of AWCD of all carbon sources was investigated. As shown in **Figure 1**, all samples showed an increase in AWCD at the early stage, indicating that microbial communities from silage samples could metabolize carbon substrates in BIOLOG ECO microplates (Miyake et al., 2016). Then, the AWCD of the raw materials exceeded 0.8, reached an inflection at 144 h after incubation, and then began to decrease (**Figure 1A**); this accords with the findings of Zhou et al. (2019). A similar pattern was observed in the silages fermented at 3 and 7 days (**Figures 1B,C**), and the value was below 0.7 when the scatter plot forms a smooth curve. Furthermore, sample 7LB showed an evidently higher metabolic rate of the substrates than the other two inoculation stages, which indicated that the utilization of substrates by 7LB was most efficient. Compared with silage at the early stage, microbial communities of samples ensilaged for 15, 30, and 60 days displayed irregular growth paths (**Figures 1D–F**), and their AWCD curves were reduced by more than 10 times.

Principal Component Analysis of Microbial Community

Figure 2 indicated the information of microbial communities with 31 carbon sources in the stable period of culture (at the time of inoculation 144 h). Principal component analysis (PCA) is a multivariate ordination method that is commonly used to analyze BIOLOG ECO data; it can differentiate the microbial communities of samples in a certain environment on different positions (**Figure 2**). Specifically, PC1 and PC2 accounted for 45.1 and 14.9% of the total variance, respectively. It is clearly discernable from **Figure 2** that the samples were divided into five groups: (i) RM (raw material), (ii) ensiling for 3 days, (iii) LP and LB ensiling for 7 days, (iv) CK ensiling for 7 days, and (v) ensiling for 15, 30, and 60 days. Only the point position of RM group was obviously clustered in one sector and distinct from the other groups, suggesting that the microbial community was substantively different from silage samples. After ensiling for 3 days, no difference was observed between treated and untreated samples, while there was slight separation in ensiling at 7 days. Interestingly, the treated and untreated samples could not be well separated from each other after ensiling for 15 days. After inoculation of microbial communities for 144 h (**Figure 3**), no difference in AWCD values between the three additives was observed except ensiling for 7 days. Sample 7LP was much smaller than 7LB (around 0.41 and 0.53, respectively).

Comparison of Metabolic Functional Diversity Indices

To further compare the metabolic functional diversity after different treatments, the Shannon diversity index (H'), Shannon evenness index (E), and Simpson index (D) after incubation for 144 h are shown in **Table 1**. Significant differences ($p < 0.05$) were found among the three indexes as a function of fermentation time. All of them exhibited analogous trends in decreasing quickly and then increasing slowly with the progression of silage time. An inflection point appeared 15 days after silage in the H' index and D index and on 7 days in the E index. This indicates that the species richness and evenness of microbial communities in paper mulberry silage were high at the early stage of fermentation and tended to decrease and then rebounded slightly during ensiling. At the inflection points of each index, both additives LB and LP increased three indexes value, and LB was higher than LP. The H' index and D index changed synchronously, indicating that species richness and the most common species in silage microbial communities in each

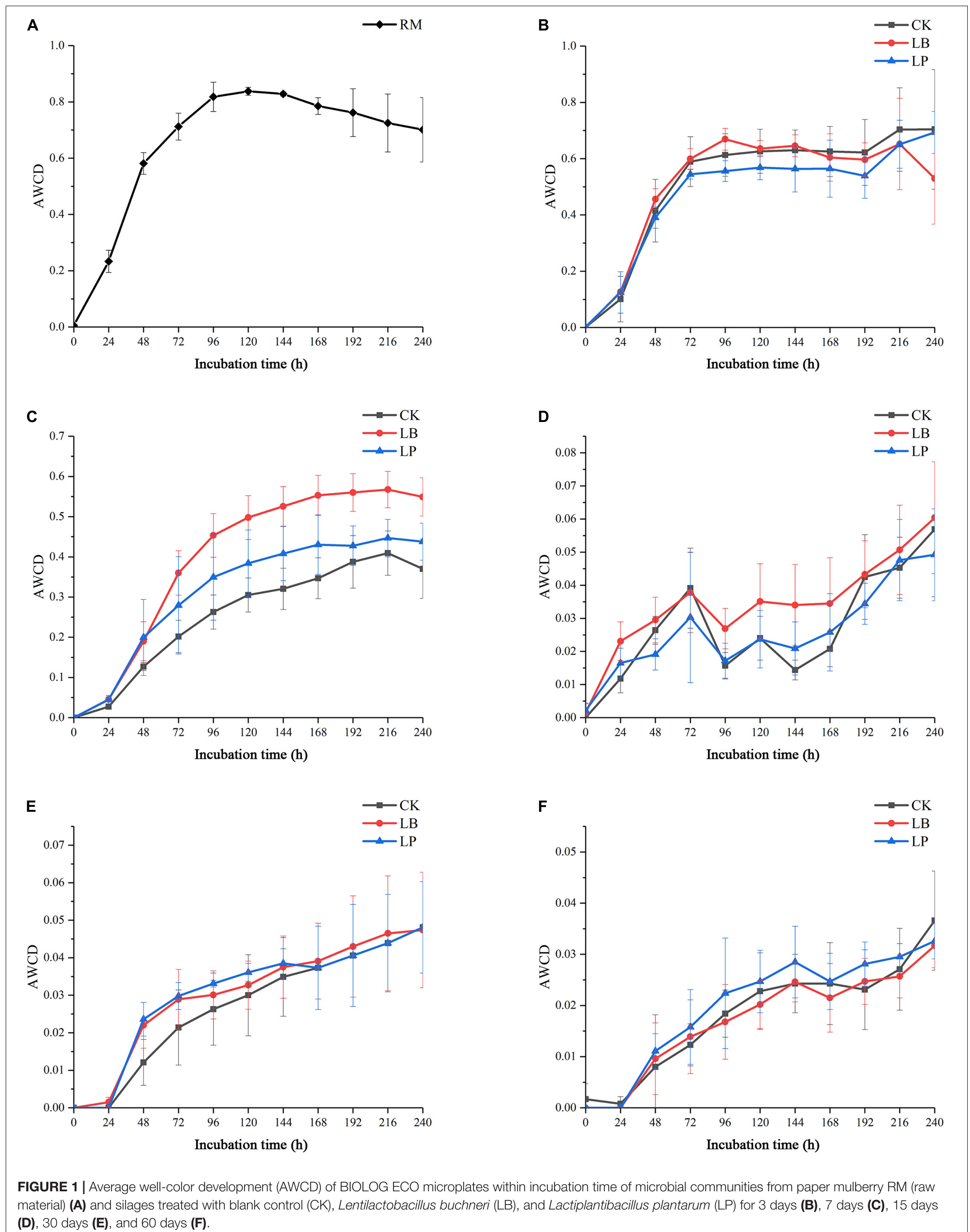

FIGURE 1 | Average well-color development (AWCD) of BIOLOG ECO microplates within incubation time of microbial communities from paper mulberry RM (raw material) **(A)** and silages treated with blank control (CK), *Lentilactobacillus buchneri* (LB), and *Lactiplantibacillus plantarum* (LP) for 3 days **(B)**, 7 days **(C)**, 15 days **(D)**, 30 days **(E)**, and 60 days **(F)**.

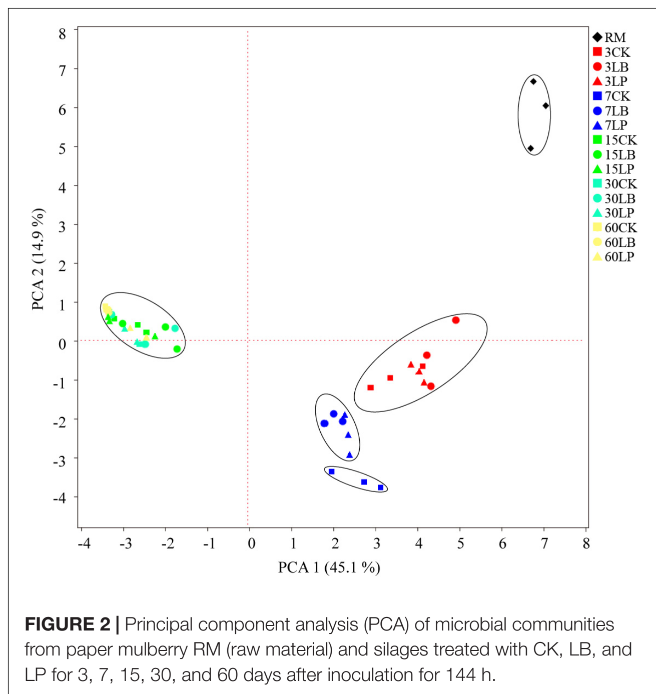

FIGURE 2 | Principal component analysis (PCA) of microbial communities from paper mulberry RM (raw material) and silages treated with CK, LB, and LP for 3, 7, 15, 30, and 60 days after inoculation for 144 h.

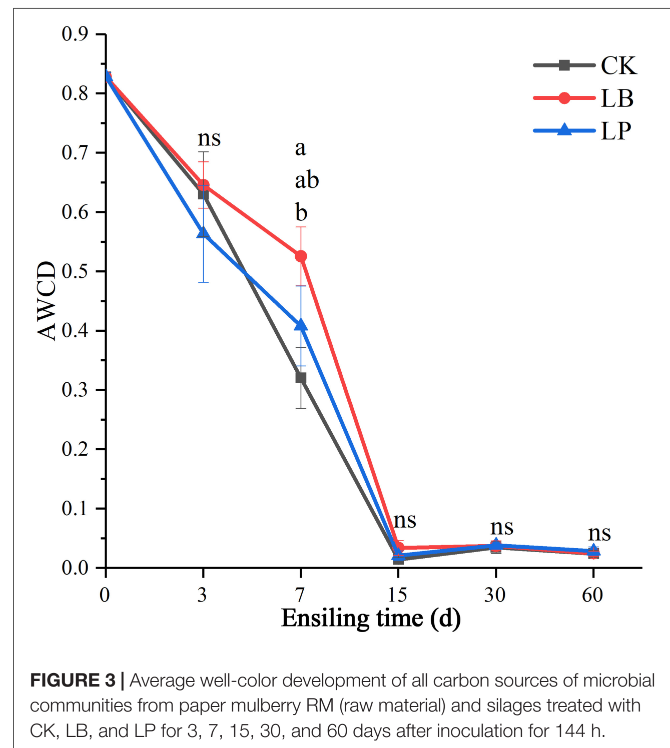

FIGURE 3 | Average well-color development of all carbon sources of microbial communities from paper mulberry RM (raw material) and silages treated with CK, LB, and LP for 3, 7, 15, 30, and 60 days after inoculation for 144 h.

treatment were closely related. With the extension of ensiling time, there was no longer a significant difference after 30 days (15 days) in the H' index and D index of CK (LB and LP).

Metabolic Utilization of Biochemical Categories Substrates

Substrate utilization profiles of the various carbon sources were classified and studied based on the average absorbance proportion of each carbon source (**Figure 4**). The results indicated that the substrate utilization profiles were different in the process of silage fermentation. In the RM, 3CK, 3LB, and 3LP samples, the six carbon sources were used to varying degrees. In the comparison of RM with ensiling for 3 days, the fractional content of carboxylic acids, amino acids, and amines/amides decreased slightly but not significantly, while the fractional content of carbohydrates increased from 24% to around 32%, suggesting that carbohydrates could be more easily utilized. With the increase in fractional content of carboxylic acids and miscellaneous for all ensiling for 7 days, the fractional content of carbohydrates for 7LB and polymers for 7CK and 7LP increased. The fractional content of miscellaneous ranked first (around 35%) in 7CK, while carbohydrates were used as the main fractional content in the 7LB (around 40%). Interestingly, in the 7LP, both miscellaneous and carbohydrates contributed almost equal contributions (around 31%). It is noteworthy that, during ensiling, the fractional content of amines/amides decreased for the first time on 7 days, and this trend occurred in carboxylic acids and amino acids on 15 days. In samples ensiled for more than 15 days, the fractional content of carbohydrates was predominant, followed by polymers (except 15CK).

Figure 5 shows the change of the AWCD associated with the six types of carbon sources as a function of ensiling time. At the beginning of ensiling, the AWCD of different substrates varied significantly. After 3 days, 3CK had the highest capability to utilize miscellaneous (a) and carbohydrates (c), while 3LP was the lowest content in both substrates. The biggest AWCD difference among the three treatments appeared in carbohydrates (c) in ensiling for 7 days, which indicated that the microbial communities of mulberry silage in the experiment caused high variations in carbohydrate metabolism with different additives. Moreover, the three treatments on carboxylic acids (d), amino acids (e), and amines/amides (f) had similar effects, with no significant difference during ensiling.

DISCUSSION

Microbial metabolism is regarded as a key link in the decomposition and transformation of nutrients in forages. Metabolism of undesirable microorganisms may lead to dry matter loss or a strong, pungent odor (Kung et al., 2018). Composed of a complex mixture of bacteria, yeasts, and molds, the microbiome associated with freshly harvested forage plays a key role in the ensiling process (Langston and Bouma, 1960). There are a variety of techniques available for assessing the differences in microbial communities during ensiling, including the plate counting method, denaturing gradient gel electrophoresis, single strand conformation polymorphisms, and other molecular approaches (Duniere et al., 2017). However, many of these classical approaches focus on characterizing the species, number, structure, and diversity of silage microbial communities but are limited in terms of metabolism. BIOLOG ECO microplate technology can competently describe metabolic

TABLE 1 | Comparison of metabolic functional diversity indices of paper mulberry silage microbial communities.

Item	Ad	Ensiling time (*T*)					SEM	*p*-Value		
		3 days	7 days	15 days	30 days	60 days		*T*	Ad	T × Ad
H′	CK	2.888[a]	2.457[b]	1.333[d]	1.814[c]	1.776[c]	0.141	<0.001	<0.001	0.049
	LB	2.984[a]	2.717[a]	1.839[b]	1.933[b]	1.753[b]				
	LP	2.926[a]	2.597[a]	1.593[b]	1.861[b]	1.883[b]				
E	CK	0.940[b]	0.900[c]	0.962[ab]	0.984[a]	0.991[a]	0.006	<0.001	0.601	0.021
	LB	0.952[ab]	0.935[b]	0.955[ab]	0.975[a]	0.979[a]				
	LP	0.943[bc]	0.932[c]	0.968[ab]	0.983[a]	0.971[ab]				
D	CK	0.940[a]	0.899[b]	0.724[d]	0.832[c]	0.828[c]	0.017	<0.001	0.001	0.003
	LB	0.945[a]	0.928[a]	0.826[b]	0.848[b]	0.819[b]				
	LP	0.942[a]	0.918[a]	0.783[b]	0.840[b]	0.840[b]				

H′, Shannon diversity; E, Shannon evenness; D, Simpson diversity; T, silage time; Ad, additives; SEM, standard error of the mean.
[a−d]Means in the same row with different superscripts differ significantly (p < 0.05).

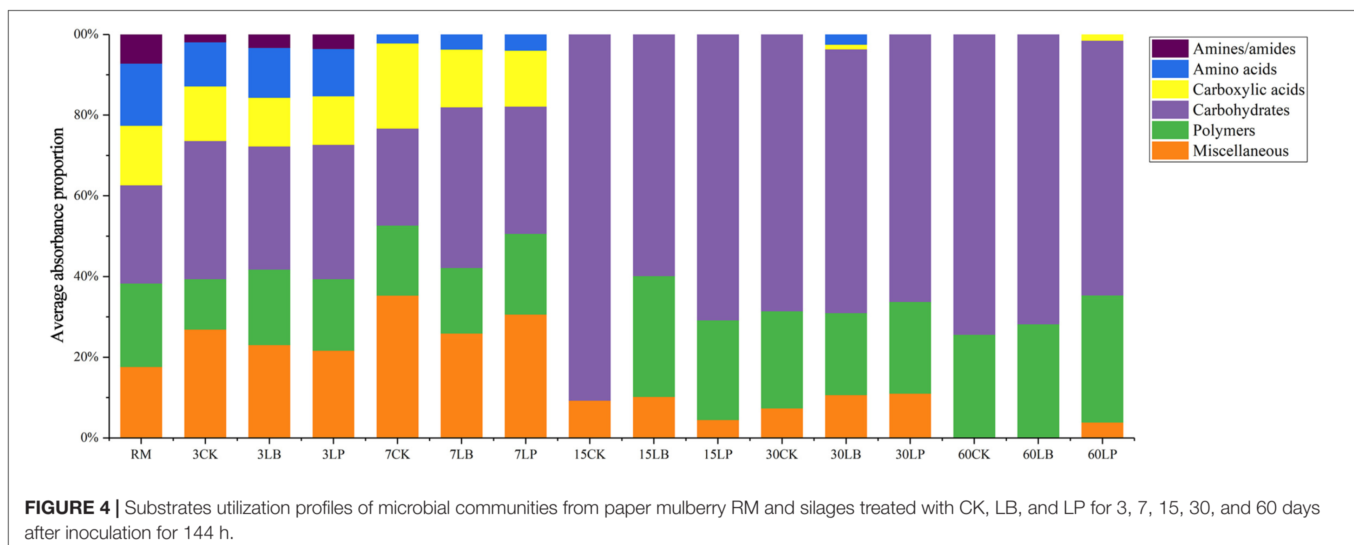

FIGURE 4 | Substrates utilization profiles of microbial communities from paper mulberry RM and silages treated with CK, LB, and LP for 3, 7, 15, 30, and 60 days after inoculation for 144 h.

functions of microbial communities, especially for environmental microorganisms (Choi and Dobbs, 1999). In this study, the microbial metabolic functions of microbial communities from paper mulberry silages were profiled with microbial metabolic activity and metabolic functional diversity indices via BIOLOG ECO microplate technology. The results suggested that this approach is feasible for studying silage microorganisms.

According to the major metabolic differences, especially the metabolism of carbohydrates, lactic acid bacteria are usually classified as obligate homofermentative, facultative heterofermentative, and obligate heterofermentative (Kandler, 1983). For making silage, LP is the most common bacterial inoculant, which was hitherto considered homofermentative but now is recognized as a taxonomically facultative heterofermentative strain (Pahlow et al., 2003). LB is the dominant species to improve aerobic stability in obligate heterofermentative additives (Kleinschmit and Jr, 2006). Due to the versatility of the BIOLOG system, it has the potential to distinguish the effects of LP and LB on metabolic activity of silage microbial communities. The AWCD decreased with fermentation time, implying that metabolic activity of silage microbial communities declined gradually. In addition, AWCD of microorganism samples from 7 days silages exhibited obviously different culture curves, listed in descending order as 7LB > 7LP > 7CK, which may be attributed to the improvement of metabolic capacity by the proliferation of lactic acid bacteria induced by additives (Siragusa et al., 2009). Homofermentative and heterofermentative additives could increase the number of lactic acid bacteria in the early stage of ensiling, and produce a large amount of lactic acid or acetic acid by metabolizing. Therefore, the low pH from lactic acid and acetic acid stabilizes silage fermentation by inhibiting the population growth and metabolic activity of spoilage microorganism intolerant of a low pH.

The substrate utilization profiles and PCA (**Figure 2**) were highly correlated. Samples separated in distinct clusters, suggesting differences in microbial community structures. This could have been caused by changes in the microbial communities of paper mulberry silage under low acidity conditions, which would be consistent with the findings of Zhang et al. (2019). As the accumulation of various organic acids increases, some species or strains reach a point where their metabolic activity drops off,

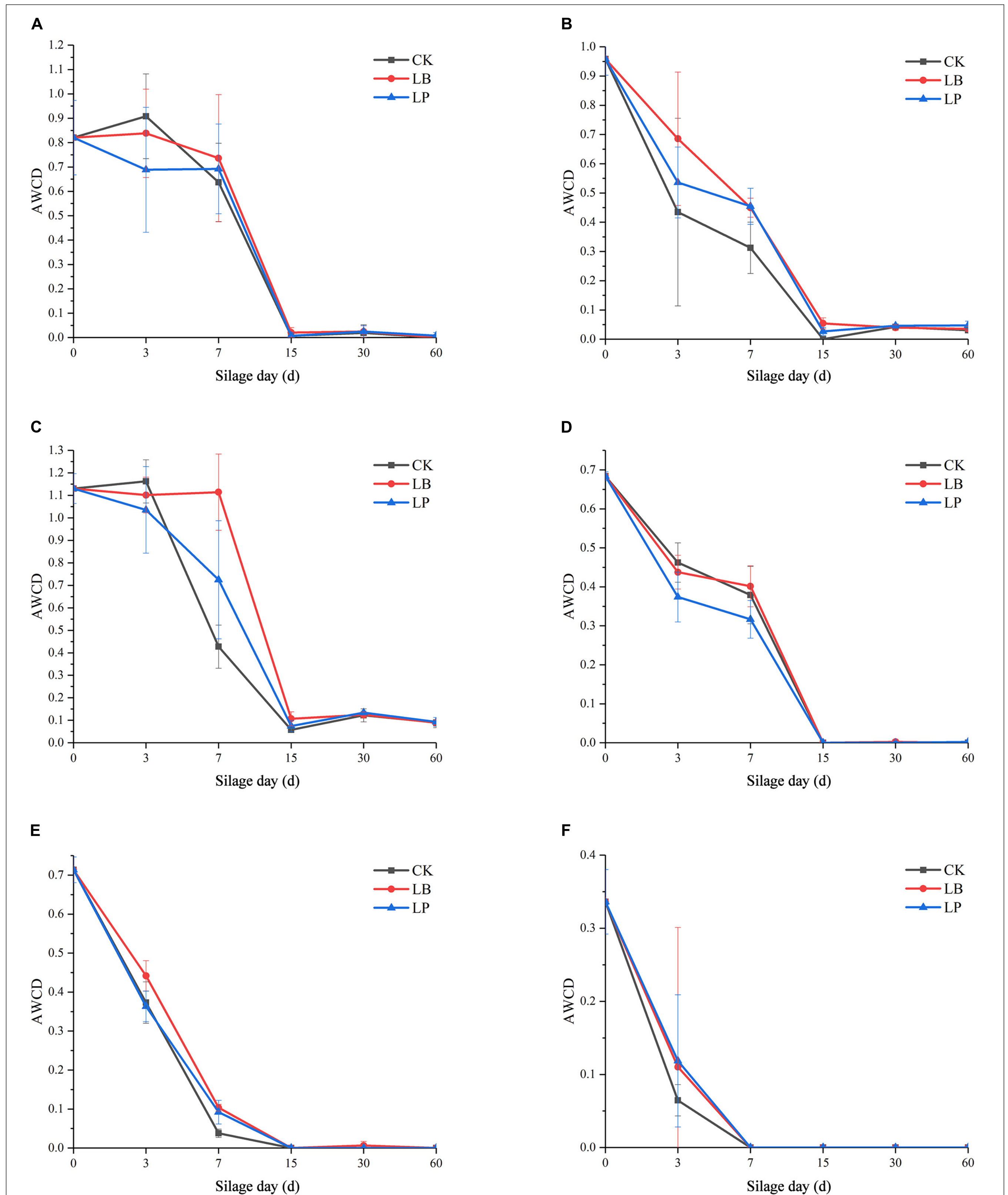

FIGURE 5 | Average well-color development of six types of carbon sources microbial communities from paper mulberry RM and silages treated with CK, LB, and LP for 3, 7, 15, 30, and 60 days after inoculation for 144 h, including miscellaneous **(A)**, polymers **(B)**, carbohydrates **(C)**, carboxylic acids **(D)**, amino acids **(E)**, and amines/amides **(F)**.

which has no impact on the effectiveness (Cai et al., 2014). This was also confirmed by higher microbial metabolic activity at the early stage than that after 15 days (**Figure 3**).

Different metabolites of microbial communities could be regarded as the final display of different lactic acid bacteria additive (Xu D. et al., 2020). Similar to previous work on the bacterial diversity of alfalfa silages as determined by sequence technology (Guo et al., 2018), ensiling led to changes in microbial community diversity in paper mulberry. This was apparent in the dynamics of the Shannon index and Simpson index, with unambiguous heterogeneities between the CK group and silages treated with LB or LP. Interestingly, lactic acid bacteria additives have previously been reported to accelerate the convergence of the microbial community during ensiling (Xu S. et al., 2020), but the opposite result was found in our study, where the three treatments showed the same trends in terms of Shannon evenness.

In previous studies, regardless of enriching lactic acid bacteria in fresh raw materials (Chen et al., 2017) or adding external lactic acid bacteria artificially during ensiling (Zhang et al., 2020), relative abundance or cultivable quantity of various species of microorganisms have been the principal consideration rather than metabolic functions. Thus, there might be some neglected effects from additives or environmental factors in the silage fermentation process. The above results support this perspective.

CONCLUSION

The present study focused on changes in microbial metabolic characteristics and functions of microbial communities in paper mulberry silages. Metabolic activities of microbial communities declined with ensiling time. After extension of the period to 15 days, the utilization of amino acids and carboxylic acids was stabilized at a low level. In addition, lactic acid bacteria additives gave evident signals on the effects of metabolic functional diversity indices. Both LB and LP accelerated the fermentation process in a steady state. Within this period, the metabolic functional diversity of LB was greater than that of LP.

By evaluating the functional diversity of silage microbial communities using the BIOLOG ECO microplates approach, this study provides insights into the effects of lactic acid bacteria additives on microbial communities' metabolic functions, whilst concomitantly offering support for the increased diffusion and use of this methodology because of its advantages in terms of ease of operation and accuracy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XW, XL, KN, and FY designed the study and wrote the manuscript. XW, XC, HL, and LG performed the experiments. XW, YL, and YX conducted the statistical analysis and visualization. KN and FY were involved in the revision of the manuscript. All authors reviewed and approved the final manuscript.

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Effects of Delayed Harvest and Additives on Fermentation Quality and Bacterial Community of Corn Stalk Silage

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This study aimed to investigate the effects of delayed harvest and additives on the fermentation quality and bacterial community of corn stalk silage in South China. The corn stalks after ear harvest at the 0 day (D0), 7 days (D7), and 15 days (D15) were used to produce small-bale silages. The silages at each harvest time were treated without (control, CK) or with *Lactobacillus plantarum* (LP) and sodium benzoate (BF). The results showed that delayed harvest increased pH and acetic acid content and reduced lactic acid content in corn stalk silage ($p < 0.05$). Compared with CK, the additives decreased the contents of butyric acid and ammonia nitrogen (NH_3-N; $p < 0.05$). The silage treated with LP increased the content of lactic acid and decreased pH ($p < 0.05$); the silage treated with BF decreased counts of coliform bacteria and yeasts and increased residual water soluble carbohydrates (WSC) content ($p < 0.05$). Single Molecule, Real-Time sequencing (SMRT) revealed that the abundance of *L. plantarum* increased, while the abundance of *Lactobacillus brevis* and *Lactobacillus ginsenosidimutans* decreased with the delayed harvest. Additives influenced the bacterial community structure of corn stalk silage, revealed by enhanced bacterial diversity on D0 and reduced on D7 ($p < 0.05$). Our research indicated that delayed harvest could exert a positive effect on acetic acid production, and additives could inhibit the butyric acid fermentation and protein degradation of corn stalk silage by shifting bacterial community composition.

Keywords: corn stalk silage, delayed harvest, sodium benzoate, *Lactobacillus plantarum*, bacterial community

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INTRODUCTION

Corn (*Zea mays*) is an important grain-forage crop. In recent years, the intercropping technology has been widely applied to produce abundant corn stalk in South China. However, most corn stalks with a high content of structural carbohydrates (50–70%) are discarded, which is wasteful and creates environmental atmospheric pollution (Li et al., 2015; Zhang et al., 2020a). Furthermore, with the increase of the demand for animal products, feed shortage hinders the development of the animal husbandry. Nowadays, high stay-green corn has been developed, which is characterized by a high chlorophyll concentration in the leaves at "stalk" maturity, with high

stalk and leaf moisture concentrations (Thomas and Smart, 1993; Bekavac et al., 1998). Importantly, moisture and crude protein (CP) concentration are relatively high in stay-green corn stalks. It is cost-effective to convert stay-green corn stalk into ruminal feed. However, how to effectively utilize corn stalks in the animal production system is a concerned issue.

Ensiling has been regarded as an effective method for preserving fresh forage due to its long storage duration, good palatability, and high nutrition. Traditional corn is usually harvested at milk ripe or wax stages for producing high-quality silage (Zhang et al., 2010; Wang et al., 2018a). In practice, delayed harvest reduced feed value of corn stalk (Cai et al., 2020). To optimize the storage of corn stalks, *Lactobacillus plantarum* (LP), a homo-fermentative strain of lactic acid bacteria (LAB), has been used as additive to enhance the fermentation quality of silage (Ni et al., 2017a; Xu et al., 2018; Guo et al., 2020a,b). Recently, sodium benzoate (BF) has been mainly applied as a microbial growth controller in silage production (Zhang et al., 2020b), which could inhibit the growth of harmful bacteria and fungi, thus improve the fermentation quality and aerobic stability of silages (Kleinschmit et al., 2005; Da Silva et al., 2015; Muck et al., 2018). However, there are only a few literatures on the efficiency of both additives (LP and BF) in producing silage of stay-green corn stalks, especially during delayed harvest in the southeast of Qinghai Tibetan Plateau.

In recent years, next-generation sequencing (NGS) technology has made the microbiota classification accurate to genus and has been increasingly utilized to analyze microbial population in silage (Ni et al., 2017a; Wang et al., 2018b; Keshri et al., 2019). However, it restricts the sensitivity and accuracy of classification. Nowadays, Single Molecule, Real-Time sequencing (SMRT) is used for high taxonomic resolution at the species level and has been employed in silages of corn, Italian ryegrass, and paper mulberry (Xu et al., 2018; Yan et al., 2019; Du et al., 2021). Nevertheless, there are few reports investigating the bacterial community based on species level and the mechanism underlying the response to additives of delayed harvest corn stalk silage.

In this study, we supposed that delayed harvest could influence the ensilability of corn stalk, and the use of additives during ensiling were beneficial to fermentation quality of the silage mainly by shifting bacterial community compositions. Therefore, we aimed to investigate the effects of delayed harvest and additives on the bacterial community and fermentation quality of corn stalk silage.

MATERIALS AND METHODS

Silage Preparation

Eighteen field plots were designed to cultivate stay-green corns (Tieyan 53, Beijing Hejiayun Agriculture Sci-Tech Co., Ltd.) on the experimental base of Sichuan Academy of Grassland Sciences, Aba, China (N 31°51'-33°33', E 101°51'-103°22'). Stay-green corn stalks were cut after 0, 7, or 15 days of ear harvest (six plots for each delayed harvest; ear provided for human diet; and assessed at D0, D7, and D15, respectively), and chopped to a length of 0.5–1.0 cm. In each plot, the chopped stalks were randomly divided into three equal parts for treatment with additive. The ensiling materials were treated with no additive as control (CK), with LP [a recommended application rate of 10^5 cfu/g fresh matter (FM), isolated from corn silage in our laboratory and was reported from Chen et al., 2020a,b] or with BF (a suggested application rate of 1.0 g/kg FM, provided from Prodpad Technology Co., Ltd.). Each bale (about one cubic meter) contained approximately 800 kg (FM) of corn stalks, and a total of 54 bales (3 delayed harvest × 3 treatments × 6 replicates) were produced using small baling system, with the density of about 580 kg/m^2 and the blank stretch film of four layers. All bales were stored at room temperature (10–20°C) for 60 days, and then sampled for the analysis on chemical, microbial composition, fermentation parameter, and bacterial community.

Chemical Analysis

Each sample of 20 g was mixed with 180 ml sterile water and a laboratory juicer for 1 min, and then filtered through four layers of cheesecloth. The filtrate was subjected to centrifugation (4,500 × *g*, 15 min, 4°C). The supernatant was used to measure pH, NH_3-N, and organic acid. The pH was determined by pH meter. NH_3-N was determined by method of Broderick and Kang (1980). Lactic, acetic, propionic, and butyric acids were analyzed using high-performance liquid chromatography (Tian et al., 2017).

Each sample of 200 g was dried at 65°C for a constant weight to determine dry matter (DM) content, and then ground by a 0.20 mm sieve for the following analysis. CP was determined by the method of AOAC (1990). Both neutral detergent fiber (aNDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash) and acid detergent fiber (ADF) were determined using an Ankom 2000 fiber analyzer (Ankom Technology, Fairport, NY) by method of Van Soest et al. (1991). WSC was determined by the method of Murphy (1958).

Microbial Analysis

The microbial count of each sample was determined by the method of Cai et al. (1999), and described by Li et al. (2020). In brief, each sample of 10 g was mixed with 90 ml sterile saline, shaken for 30 min and then filtered through sterile gauze. Serial dilutions were performed. The count of LAB was determined on MRS agar (CM188, Land Bridge Technology Co., Ltd., Beijing, China) and incubated at 30°C for 48–72 h under anaerobic conditions (Anaerobic box; TEHER Hard Anaerobox, ANX-1; Hirosawa Ltd., Tokyo, Japan). Aerobic bacteria were counted on nutrient agar (CM107, Land Bridge Technology Co., Ltd., Beijing, China) and inoculated at 28°C for 24–36 h under aerobic conditions. Coliform bacteria were counted on ECCA cheomogenic medium (RP0436, Guangzhou LES Biological Technology, Co. Ltd., Guangzhou China). Yeasts and molds were counted on malt extract agar with 1.5 mg/L Tetracycline (CM164, Land Bridge Technology Co., Ltd., Beijing, China) and incubated at 28°C for 48 h. Yeasts were distinguished from molds through colony appearance and observation of cell morphology.

Total genome DNA from each sample was extracted by CTAB method. DNA after purification was diluted to 1 ng/ml using sterile water. The full-length 16S ribosomal RNA (rRNA) gene was amplified used specific primer (27F and 1541R) with the barcode (Yan et al., 2019). The PCR reaction was carried out by TransStart®FastPfu DNA Polymerase (TransGen Biotech). Triplicate amplifications from each sample were mixed for establish libraries. PCR products were mixed in equal density ratios and purified with QIAquick@Gel Extraction Kit (QIAGEN). Libraries were established using SMRTbellTMTemplate Prep Kit (PacBio) following manufacturer's recommendations, and then sequenced on the PacBio Sequel platform.

Raw sequences were initially processed through the PacBio SMRTportal. Sequences were filtered to produce reads without barcode and primer sequence. The reads were compared with the reference database using UCHIME algorithm[1] to detect chimera sequences (Edgar et al., 2011), and then the chimera sequences were removed for obstaining clean reads (Haas et al., 2011). Sequences analysis was performed by Uparse software (Uparse v7.0.1001; Edgar, 2013).[2] Sequences with the similarity ≥97% were distributed to the same operational taxonomic unit (OUT).

Following the OTU analysis, principal coordinates analysis (PCoA) was performed using R software (Version 2.15.3) based on the beta-diversity analysis. Representative sequence for each OTU was screened out for annotating taxonomic information in the SSUrRNA Database of Silva Database (Qiong et al., 2007; Christian et al., 2012). After the establishment of the phylogenetic relationship (Edgar, 2004), the number of observed species, richness index of abundance-based coverage estimator (ACE) and Chao 1, and diversity index of Shannon were calculated using QIIME software (Version1.9.1) and displayed with R software (Version 2.15.3). The heat map of spearman analysis was performed using a R based statistics tool.

Statistical Analysis

Before statistical analysis, microbial counts of each silage sample were estimated as $\log_{10}$ cfu/g of FM. Factorial analysis of variance was performed to evaluate the effects of delayed harvest (D), additive (A), and their interaction (D × A) on the chemical composition, microbial population, and bacterial community indices of silage in the General Line Model of SPSS (SPSS 25.0 program, SPSS Inc., Chicago, Illinois, United States). There were significant differences only when the probability level was lower than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

Chemical and Microbial Compositions of Corn Stalks Prior to Ensiling

Chemical and microbial compositions of stay-green corns prior to ensiling were shown in **Table 1**. Delayed harvest had significant effects on contents of DM, WSC, CP, aNDF, ADF, and counts of LAB, coliform bacteria, aerobic bacteria, and yeasts ($p < 0.05$). DM, aNDF, and ADF contents of corn stalks significantly increased with the harvest delayed ($p < 0.05$), which indicated that the lignification degree of corns could be deepened with delayed harvest. WSC and CP contents in this study significantly decreased from 8.88 to 7.29% DM and from 9.22 to 6.53% DM, respectively. There was also similar observation from Guo et al. (2019), which suggested a loss of nutrient in delayed harvest corn stalk. WSC is an important substance for the growth and propagation of epiphytic microorganisms, especially for LAB. In this study, the WSC content beyond 6% DM was sufficient for the production of high-quality silage. However, relatively high counts (10^5–10^7 cfu/g FM) of undesirable microorganisms (coliform bacteria, aerobic bacteria, and yeasts) on the plants were observed on corn stalks from D0 to D15. Although the count of epiphytic LAB on the plant of direct-cut stay-green corn (D0) was 10^5 cfu/g FM, which was enough for initiating lactic acid fermentation under anaerobic condition. The count decreased from D0 to D15 to 10^4 cfu/g FM, which was insufficient for silage preservation (Cai et al., 1999). The above findings indicated that the delayed harvest could be harmful for the fermentation quality of corn stalk silage, and the unfavorable properties of stay-green corns for silage implied that it was necessary to add exogenous additives to enhance fermentation during ensiling.

Chemical Composition of Corn Stalk Silage

The chemical composition of corn stalk silages treated without (CK) or with LP and BF was shown in **Table 2**. Delayed harvest significantly affected all parameters of chemical composition ($p < 0.001$). Additives had significant effects on residual WSC, aNDF, and ADF contents ($p < 0.05$). Their interaction only had a significant effect on residual WSC and aNDF contents of silage ($p < 0.05$). DM, aNDF, and ADF contents of silage significantly increased with advancing maturity, which might due to the decrease of the moisture and the increase of the cell wall content in stem with advancing maturity (Yari et al., 2012; Sikora et al., 2019). The CP content of corn stalk silage gradually decreased ($p < 0.001$) from D0 to D15, which might due to the higher protein content in corn leaves than stems. Moreover, a high CP content was found in LP-treated silage numerically. The behind reason might be that proteolysis was inhibited by acid accumulation during ensiling (Chen et al., 2020b). During ensiling, some microbes, such as coliform bacteria, LAB, and yeasts, could produce energy with WSC (Kung et al., 2018; Queiroz et al., 2018). In this study, a high residual WSC content (3.48–4.64% DM) was observed in BF-treated silages on D0 and D15 ($p < 0.05$). Similar reports were also found from Alli et al. (1985) and Da Silva et al. (2015), and that might because the antimicrobial capacity of BF could inhibit the growth of microbe, and then reduce their WSC metabolism. This confirmed that the treatment with LP and BF could enhance the preservation of silage nutrients although there was a loss of nutrients with the delayed harvest.

[1]http://www.drive5.com/usearch/manual/uchime_algo.html
[2]http://drive5.com/uparse/

TABLE 1 | Chemical and microbial compositions of corn stalks after 0 (D0), 7 (D7), and 15 (D15) days of ear harvest.

Delayed harvest	DM	WSC	CP	aNDF	ADF	Lactic acid bacteria	Coliform bacteria	Aerobic bacteria	Yeasts	Molds
	%	% DM				Log$_{10}$ cfu/g FM				
D0	25.31^{c}	8.88^{a}	9.22^{a}	50.41^{b}	31.18^{b}	5.35^{a}	6.50^{a}	7.48^{a}	6.11^{b}	3.52
D7	28.46^{b}	8.15^{ab}	7.01^{b}	58.79^{a}	32.19^{b}	4.87^{b}	5.23^{b}	7.16^{a}	6.68^{a}	3.44
D15	35.18^{a}	7.29^{b}	6.53^{c}	61.48^{a}	34.01^{a}	4.19^{c}	5.76^{b}	5.45^{b}	6.82^{a}	3.69
SEM	1.49	0.35	0.42	1.69	0.47	0.18	0.20	0.33	0.12	0.09
Significance (*p*-value)	<0.001	0.044	<0.001	<0.001	0.007	0.001	0.003	<0.001	0.006	0.658

DM, dry matter; WSC, water soluble carbohydrates; CP, crude protein; ADF, acid detergent fiber; aNDF, neutral detergent fiber; FM, fresh matter; and SEM, standard of error mean. Different letters within a column are significantly different (p < 0.05).

TABLE 2 | Chemical composition of corn stalk silages treated without (CK) or with *Lactobacillus plantarum* (LP) and sodium benzoate (BF).

Delayed harvest	Additive	DM	Residual WSC	CP	aNDF	ADF
		%	% DM			
	CK	23.89	4.02^{bc}	8.59	52.75^{f}	34.17
D0	LP	22.20	4.32^{ab}	9.02	56.66^{e}	33.11
	BF	22.93	4.64^{a}	8.58	53.94^{f}	32.58
	CK	26.01	2.78^{e}	6.09	62.15^{bc}	34.12
D7	LP	27.81	3.67^{cd}	6.11	61.16^{cd}	33.28
	BF	26.76	3.48^{d}	6.10	59.01^{d}	33.03
	CK	32.04	2.35^{f}	5.24	64.84^{a}	36.32
D15	LP	34.05	2.30^{f}	5.44	65.44^{a}	36.92
	BF	32.46	3.94^{bc}	5.42	63.77^{ab}	35.88
SEM		4.37	0.12	0.21	0.65	0.24
Significance (*p*-value)						
Delayed harvest		<0.001	<0.001	<0.001	<0.001	<0.001
Additive		0.581	<0.001	0.402	0.008	0.014
Delayed harvest × additive		0.762	<0.001	0.760	0.034	0.313

DM, dry matter; Residual WSC, residual water soluble carbohydrates; CP, crude protein; ADF, acid detergent fiber; aNDF, neutral detergent fiber; and SEM, standard of error mean. D0-15 indicated stay-green corn stalks after 0, 7, and 15 days of ear harvest. Different letters within a column are significantly different (p < 0.05).

As shown in **Table 3**, the interaction of delayed harvest and additives significantly affected final pH and the contents of NH_3-N, lactic acid, acetic acid, and butyric acid ($p < 0.05$), in which delayed harvest had significant effects on all the fermentation indicators in **Table 3** ($p < 0.05$), and additives significantly affected final pH and the contents of NH_3-N, lactic acid, and acetic acid ($p < 0.001$). According to Kung et al. (2018), the concentrations of lactic acid and acetic acid were usually negatively correlated to DM content. Our study showed that delayed harvest significantly decreased the lactic acid content ($p < 0.001$), but increased acetic acid content with the increase of DM content ($p < 0.001$). The probable cause was that delayed harvest promoted the metabolism of some acetic acid-producing microbes.

When the DM content of fresh forages was above 30–35% DM and induced the rapid production of lactic acid, clostridial fermentation could be minimized because clostridia were intolerant of high osmotic pressure and low pH, thus resulting in a content of low butyric acid (Kung et al., 2018). In our study, the content of butyric acid decreased with the harvest delayed ($p < 0.05$), due to the increase of DM content. Additives significantly reduced the content of butyric acid on D0 and D7 ($p < 0.05$). This might be due to the lower pH in LP-treated silage and the antibacterial ability of BF. NH_3-N is an important indicator for CP degradation, and its content in well-preserved silage should be lower than 10% total N (Chen et al., 2020b; Cheng et al., 2021). However, the NH_3-N content of CK on each harvest day was higher than that level. The addition of BF and LP reduced the NH_3-N content in silages on D0 and D7 compare with the CK samples ($p < 0.05$). Furthermore, the NH_3-N content of LP-treated silage on D0 and BF-treated silages on D0 and D15 were below 10% total N, which suggested their low protein proteolysis.

Delayed harvest restricted the reduction of pH value, which was similar to Wang et al. (2018a), because high DM and low moisture contents in corn stalks after delayed harvest inhibited the silage fermentation. In general, the pH required for successful ensiling was lower than 4.2. In this study, the pH in the LP-treated silages was below 4.2, which might indicate that the addition with LP could help inhibit the unfavorable factors produced by the lower fermentation quality due to delayed harvest. Compared with CK samples, the treatment with LP significantly increased the content of lactic acid in silages on D7 and D15 ($p < 0.05$). Therefore, LAB inoculation might help promote rapid and vigorous fermentation of corn stalks. Studies from Kleinschmit et al. (2005), Teller et al. (2012), and Da Silva et al. (2015) showed that the addition of BF during ensiling had no effect on the concentrations of lactic acid in corn silages. In this study, the BF-treated silage had the highest pH value and the lowest content of lactic acid ($p < 0.05$), which further confirmed that BF might inhibit the activity or growth of microbial groups, thus reducing the fermentation to acid production. This indicated that good nutrient preservation of LP- or BF-treated silages were attributed to different fermentation qualities. Inoculation of LP during ensiling exerted a good performance in improving silage fermentation, whereas the use of BF during ensiling reduced NH_3-N by limiting the microbial fermentation.

Microbial Population of Corn Stalk Silage

The silage fermentation process is initiated and controlled with microorganisms. As shown in **Figure 1**, all silages showed a similar microbial composition by plate culture. The counts of yeasts and coliform bacteria only in BF-treated silages were

TABLE 3 | Final pH, NH_3-N, and fermentation acids of corn stalk silages treated without (CK) or with LP and BF.

Delayed harvest	Additive	Final pH	NH_3-N	Lactic acid	Acetic acid	Propionic acid	Butyric acid
			% total N	% DM			
	CK	3.73[e]	13.32[a]	2.60[ab]	0.15[e]	0.12	0.19[a]
D0	LP	3.70[e]	8.77[c]	2.79[a]	0.19[de]	0.14	0.11[b]
	BF	3.89[d]	8.91[c]	2.70[a]	0.17[de]	0.14	0.13[b]
	CK	4.41[a]	13.41[a]	1.04[e]	0.38[b]	0.05	0.13[b]
D7	LP	4.11[c]	10.21[b]	2.40[b]	0.23[c]	0.07	0.01[c]
	BF	4.31[ab]	10.21[b]	0.96[e]	0.22[cd]	0.06	0.01[c]
	CK	4.20[bc]	10.80[b]	1.30[d]	0.46[a]	0.14	0.01[c]
D15	LP	4.18[bc]	10.57[b]	2.06[c]	0.37[b]	0.15	0.01[c]
	BF	4.36[a]	8.94[c]	1.33[d]	0.40[ab]	0.10	0.01[c]
SEM		0.27	0.25	0.06	0.02	0.04	–
Significance (*p*-value)							
Delayed harvest		<0.001	<0.001	<0.001	<0.001	<0.001	0.007
Additive		<0.001	<0.001	<0.001	<0.001	0.393	0.428
Delayed harvest × additive		0.028	<0.001	<0.001	<0.001	0.322	0.006

DM, dry matter; NH_3-N, ammonia nitrogen; and SEM, standard of error mean. D0-15 indicated corn stalks after 0, 7, and 15 days of ear harvest. Different letters within a column are significantly different ($p < 0.05$).

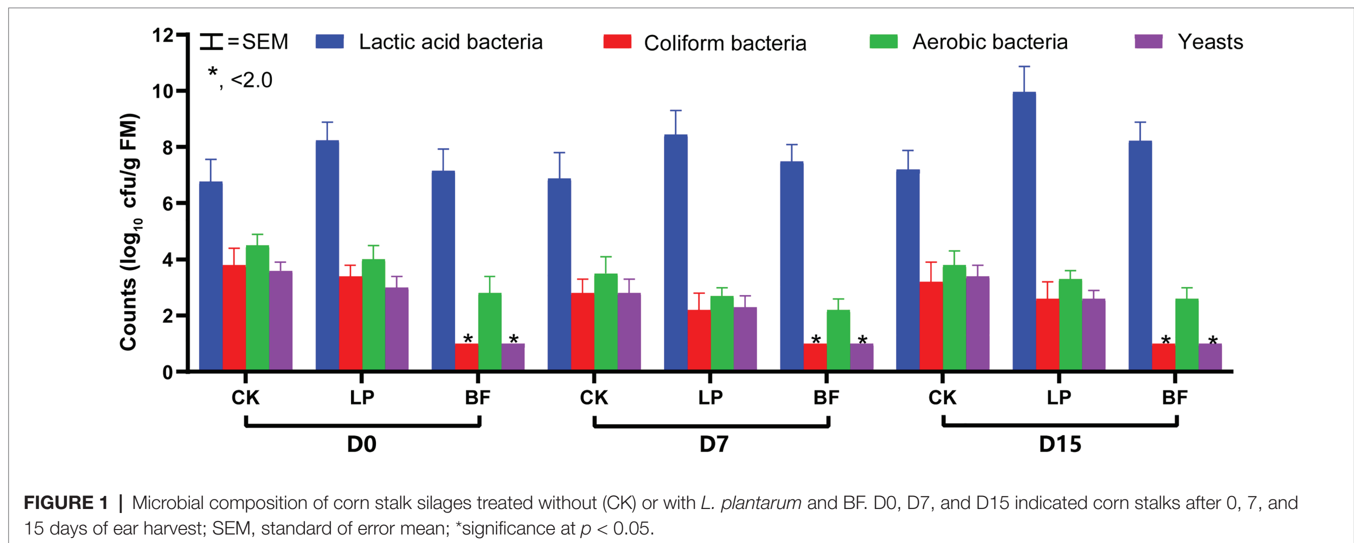

FIGURE 1 | Microbial composition of corn stalk silages treated without (CK) or with *L. plantarum* and BF. D0, D7, and D15 indicated corn stalks after 0, 7, and 15 days of ear harvest; SEM, standard of error mean; *significance at $p < 0.05$.

below the detected level (<2.0 $\log_{10}$ cfu/g FM). The inhibition of yeasts in BF-treated silages might involve some mechanisms of defect in amino acid uptake, such as the failure of enzymatic systems in the glycolysis and citric acid cycle in yeast cell (Santos et al., 2019). The similar results occurred in grain and corn silages (Knicky and Spörndly, 2011; Morais et al., 2017; Santos et al., 2019). According to Kung et al. (2018), coliform bacteria and yeasts could compete with LAB for fermentation substrates. Our findings indicated that the treatment with BF was useful to inhibit yeasts and coliform bacteria to create the condition where LAB population could grow and propagate quickly.

Bacterial Community Indices of Corn Stalk Silages

The third generation Pacific Biosciences (PacBio) SMRT improves the classification sensitivity and accuracy of microbial community in silages (Xu et al., 2018). In order to further investigate the bacterial community of corn stalk silage after delayed harvest in South China, SMRT sequencing was used to describe them.

The bacterial alpha diversity of silage samples was shown in **Table 4**. The PE reads of each sample ranged from 5,847 to 6,693, and the total number of observed species was 875. The delayed harvest, additives and their interactions significantly affected the bacterial richness (Chao 1 and ACE) and the diversity (Shannon and PD whole tree; $p < 0.05$). Compared with CK samples, additives increased Chao 1, ACE, Shannon, and PD whole tree of silages on D0 ($p < 0.05$). However, the reverse trend was observed in silages at D7 and D15. This might be due to the fact that the use of additives enhanced the superiority of dominant bacteria and subsequently reduced the richness and diversity of other bacteria in silages with a high DM (Ni et al., 2017a). Especially, the treatment with LP had the lowest Shannon and PD whole tree in silages on D7 and D15 ($p < 0.05$). According to Kung et al. (2003) and

TABLE 4 | Bacterial alpha-diversity of corn stalk silages treated without (CK) or with *L. plantarum* and BF.

Delayed harvest	Additive	PE reads	Observed species	Shannon	Chao 1	ACE	PD whole tree
	CK	6,050	110[bc]	2.27[b]	214.00[d]	239.01[d]	8.67[cd]
D0	LP	6,043	221[a]	2.76[a]	492.73[b]	594.48[b]	12.94[a]
	BF	6,185	157[ab]	2.76[a]	323.83[c]	397.76[c]	10.58[b]
	CK	6,428	134[b]	1.76[c]	576.85[a]	747.02[a]	9.73[bc]
D7	LP	5,847	37[c]	0.91[e]	51.87[f]	55.06[f]	4.95[fg]
	BF	6,165	86[bc]	1.21[d]	130.29[e]	150.80[e]	8.27[d]
	CK	6,390	55[c]	2.26[b]	64.30[f]	68.71[f]	6.36[e]
D15	LP	6,693	30[c]	1.16[d]	37.76[f]	38.23[f]	4.39[g]
	BF	6,049	45[c]	1.88[c]	54.22[f]	58.76[f]	6.01[ef]
SEM		257	9	0.09	26.58	34.06	0.39
Significance (*p*-value)							
Delayed harvest		0.327	<0.001	<0.001	<0.001	<0.001	<0.001
Additive		0.720	0.704	<0.001	<0.001	<0.001	0.031
Delayed harvest × additive		0.443	<0.001	<0.001	<0.001	<0.001	<0.001

SEM, standard of error mean. D0-15 indicated stay-green corn stalks after 0, 7, and 15 days of ear harvest. Different letters within a column are significantly different (p < 0.05).

Oliveira et al. (2017), the inoculation with exogenous LAB generally had a positive effect on sufficient lactic acid production and inhibited the activity of other harmful bacteria during the fermentation process. The findings of our study might confirm that exogenous *L. plantarum* exhibited high competitiveness for dominance during silage fermentation of corn stalks. Therefore, it is necessary to investigate the dominated species of bacterial community of corn stalks.

As shown in **Figure 2**, PCoA revealed that component 1 and component 2 could explain 68.84 and 10.59% of the total variance in bacterial community structure, respectively. Silage samples were well separated between D0, D7, and D15, which suggested that bacterial community would be affected by harvest time of corn stalks. Report from Ni et al. (2017b) showed that the variation of microbial community might explain the differences of silage quality. The findings of our study revealed that additives increased the dissimilarity in bacterial community in silage samples on D7 and D15, suggesting that the treatment with LP and BF could markedly shift the bacterial community composition and construction.

The relative abundance of the top 10 bacterial species in corn stalk silage was shown in **Figure 3**. *Lactobacillus plantarum* and *Lactobacillus brevis* were the dominant species in corn stalk silages. With the delayed harvest, the abundance of *L. plantarum* increased, while the abundance of *L. brevis* decreased. It is generally recognized that *L. plantarum* is often used as an inoculant to promote silage fermentation (Yan et al., 2019). However, the increased dominance of *L. plantarum* was not effective in enhancing the content of lactic acid with the delayed harvest, because the low moisture content inhibited the activity of most microorganisms, and dominant *L. plantarum* showed a high survival competitiveness but a limited fermentation capacity on inferior condition. Compared with CK samples, the abundance of *L. brevis* was lower, while the abundance of *L. plantarum* was higher in additive-treated silage on D15, which indicated that additives could promote the dominance of *L. plantarum* in silage with a high DM. *Weissella* is abundant in fresh materials and tended to be dominated in the early ensiling period, while they will be replaced by more acid-tolerant *Lactobacillus* in the late ensiling period (Graf et al., 2016; Pereira et al., 2019).

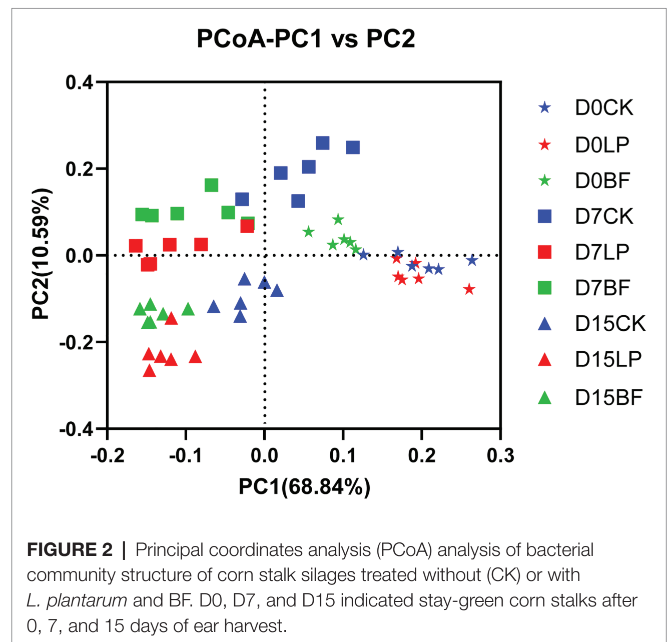

FIGURE 2 | Principal coordinates analysis (PCoA) analysis of bacterial community structure of corn stalk silages treated without (CK) or with *L. plantarum* and BF. D0, D7, and D15 indicated stay-green corn stalks after 0, 7, and 15 days of ear harvest.

In our study, the high abundance of *Weissella paramesenteroides* in CK and BF treatment of silages on D7 might be due to the slow fermentation process, resulting in a lower abundance of *L. plantarum*. Furthermore, *Lactobacillus ginsenosidimutans* and *Lactobacillus spicheri* could be isolated from fermented food, and sometimes used as probiotic strains for improvement of functional foods (Gautam and Sharma, 2015; Chiş et al., 2020). However, their effects on silages should be further studied.

Correlation Between Silage Parameters and Bacterial Community

The spearman analysis between silage parameters and bacterial species was shown in **Figure 4**. The results showed that residual WSC was negatively correlated to *L. plantarum* ($p < 0.05$), which confirmed that WSC was a determinant substrate for *L. plantarum* in silage fermentation (Chen et al., 2020b).

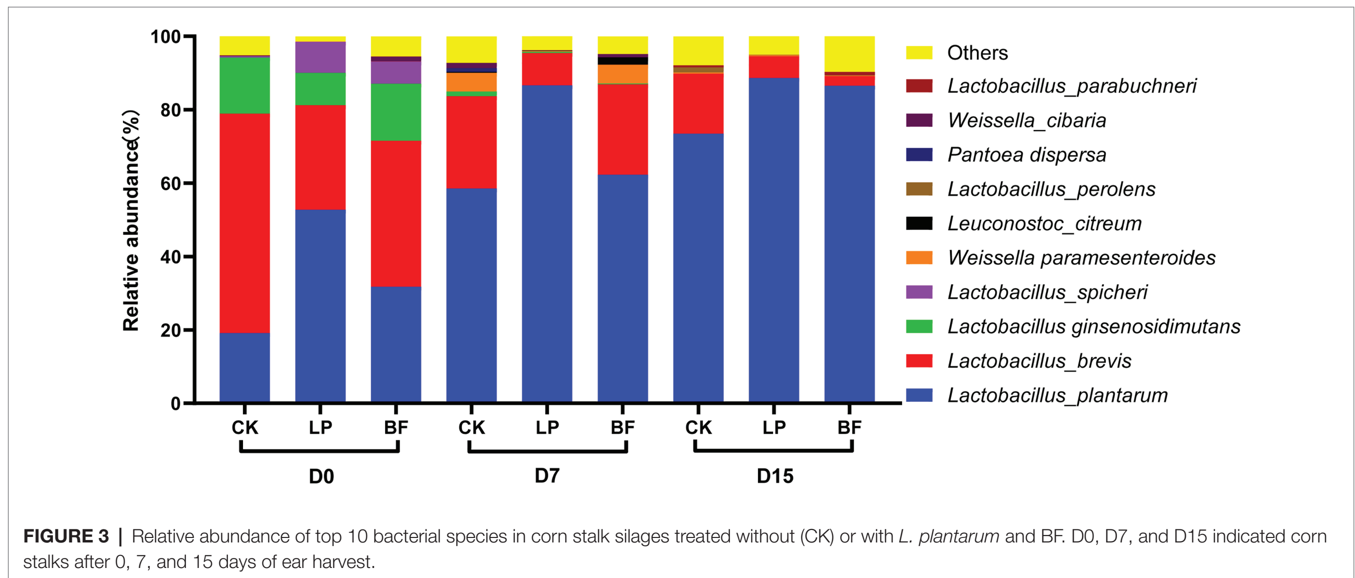

FIGURE 3 | Relative abundance of top 10 bacterial species in corn stalk silages treated without (CK) or with *L. plantarum* and BF. D0, D7, and D15 indicated corn stalks after 0, 7, and 15 days of ear harvest.

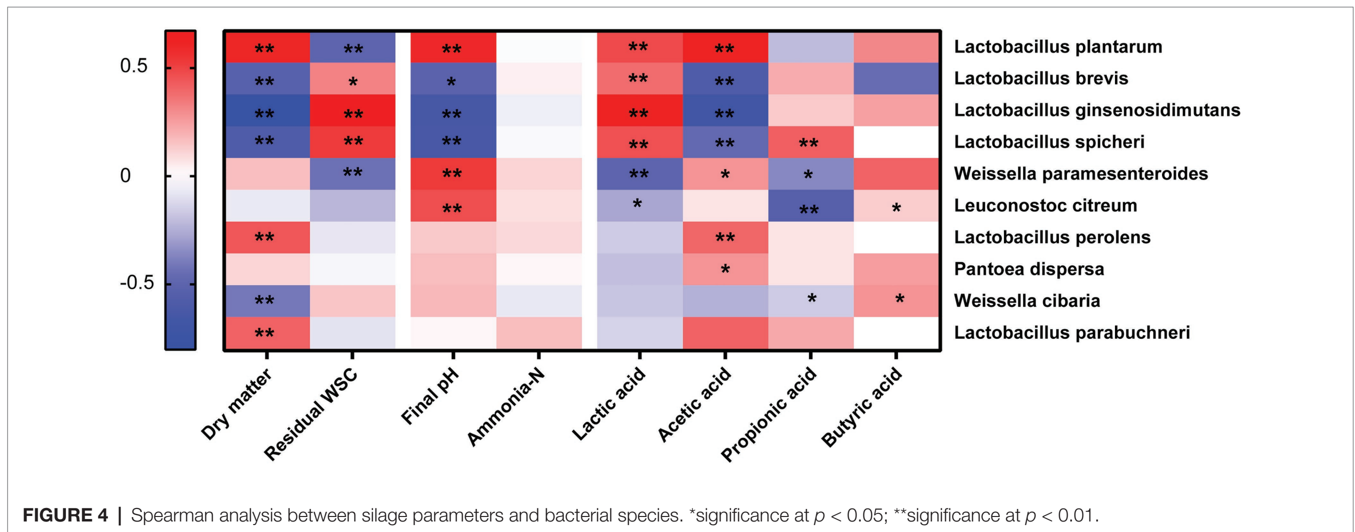

FIGURE 4 | Spearman analysis between silage parameters and bacterial species. *significance at $p < 0.05$; **significance at $p < 0.01$.

Lactobacillus exerted a significant effect in increasing lactic acid and reducing pH in the later stage (Cai et al., 1998). Therefore, our study showed that lactic acid was positively correlated to some *Lactobacillus* genera (*L. plantarum*, *L. ginsenosidimutans*, and *L. spicheri*; $p < 0.05$). It was generally recognized that *L. plantarum* was the dominant homo-fermentative bacteria for increasing lactic acid and decreasing pH in silage fermentation. However, the final pH and acetic acid were positively correlated to *L. plantarum* ($p < 0.05$). In addition, the dry matter content was also positively correlated to *L. plantarum* in this study ($p < 0.05$). These were consistent with the results of silage quality and bacterial community of corn stalk silage after the delayed harvest, that is, the DM content and *L. plantarum* abundance increased, while the lactic acid content decreased, and the acetic acid content enhanced. The similar results were also reported by Ni et al. (2017a), who found that the lactic acid/acetic acid was lower in samples with a high DM compared with that in soybean silage samples with a low DM. That might because the high DM content was more likely cause the metabolism of hetero-fermentative *L. plantarum*, but more studies should be further conducted.

CONCLUSION

Delayed harvest could restrict lactic acid fermentation, and increase acetic acid production and pH value in corn stalk silage. LP and BF additives have an ability of improving the fermentation quality of corn stalk silage through decreasing butyric acid and NH_3-N contents. In addition, SMRT result showed that LP and BF could enhance fermentation quality of delayed harvest corn stalk by shifting bacterial community. Overall, our research confirmed that inoculated with LP or BF could be feasible ways for improving the delayed harvest corn stalk silage.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in Sequence Read Archive (SRA), http://www.ncbi.nlm.nih.gov/sra/, PRJNA718962.

AUTHOR CONTRIBUTIONS

LG, YL, PL, LC, and WG designed the study and wrote the manuscript. PL and LC performed the experiments. LG and PL conducted the statistical and bioinformatics analysis. PL and CZ were involved in the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Influence of Pyroligneous Acid on Fermentation Parameters, CO_2 Production and Bacterial Communities of Rice Straw and Stylo Silage

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Carbon dioxide (CO_2) is a primary greenhouse gas and the main cause of global warming. Respiration from plant cells and microorganisms enables CO_2 to be produced during ensiling, a method of moist forage preservation applied worldwide. However, limited information is available regarding CO_2 emissions and mitigation during ensiling. Pyroligneous acid, a by-product of plant biomass pyrolysis, has a strong antibacterial capacity. To investigate CO_2 production and the influence of pyroligneous acid, fresh stylo, and rice straw were ensiled with or without 1% or 2% pyroligneous acid. Dynamics of the fermentation characteristics, CO_2 production, and bacterial communities during ensiling were analyzed. Pyroligneous acid increased the lactic acid content and decreased the weight losses, pH, ammonia-N content, butyric acid content, and coliform bacterial numbers (all $P < 0.05$). It also increased the relative abundance of *Lactobacillus* and decreased the relative abundances of harmful bacteria such as *Enterobacter* and *Lachnoclostridium*. Adding pyrolytic acids reduced the gas production, especially of CO_2. It also increased the relative abundances of CO_2-producing bacterial genera and of genera with the potential for CO_2 fixation. In conclusion, adding pyroligneous acid improved the fermentation quality of the two silages. During ensiling, CO_2 production was correlated with bacterial community alterations. Using pyroligneous acid altered the bacterial community to reduce CO_2 production during ensiling. Given the large production and demand for silage worldwide, application of pyroligneous acid may be an effective method of mitigating global warming via CO_2 emissions.

Keywords: greenhouse gas, bacterial community, rice straw, stylo, fermentation quality

INTRODUCTION

Carbon dioxide (CO_2), a primary greenhouse gas, has received increasing attention in the past two decades and has become a priority because of its low-carbon and sustainable development worldwide (Ray et al., 2019). Approximately, 15% of anthropogenic greenhouse gas emissions are generated by animal husbandry production (Adegbeye et al., 2019). In recent years, considerable efforts have been made to reduce greenhouse gas emissions from animal husbandry production and manure treatment. Ensiling is a traditional method of conserving forage, and silage is used as an important nutrient feed source for ruminants worldwide (Dunière et al., 2013). In China, silage production is reported to exceed 280 million tons annually (Liu Z. et al., 2020) and is expected to further increase as the consumption of livestock products, such as milk and beef, increases. Metabolism of microorganisms and plant cells in silage leads to gas emissions, of which, CO_2 is the main gas produced. Cai et al. (1997) reported that after 60 days of fermentation, gas production exceeds 6.0 L/kg of fresh matter. However, McEniry et al. (2011) reported that CO_2 production mainly occurs in the early stages of ensiling, which constitutes > 60% of all the gas produced. CO_2 production during ensiling leads to nutrient loss from the silage and impacts the greenhouse effect, which affects the earth's ecology. However, little research has been conducted on CO_2 emissions from silage.

Pyroligneous acid, a by-product of plant biomass pyrolysis, is a complex, condensed, crude, and highly oxygenated aqueous liquid fraction generated during wood charcoal production (Li et al., 2018). Pyroligneous acid consists of more than 200 compounds, including furan, organic acids, esters, phenols, alcohols, and pyran derivatives (Liu X. et al., 2020), and is recycled in many areas as an important commercially valuable resource. Pyroligneous acid is beneficial to agriculture and has been used as an insecticide, fertilizer, soil enhancer, animal feed supplement, and source of smoke flavoring for food (Zheng et al., 2020). Pyroligneous acid has strong antibacterial abilities owing to the presence of organic acids and phenolic compounds. Previous studies have shown that pyroligneous acid prevents the activities of microorganisms such as *Pseudomonas*, *Escherichia*, *Staphylococcus*, *Aspergillus*, and *Candida*, which are abundant during ensiling (de Souza Araújo et al., 2018; Suresh et al., 2019; Bai et al., 2020; Wu et al., 2020). However, the effects of pyroligneous acid on bacterial communities during ensiling remain unknown.

We hypothesized that adding pyroligneous acid during ensiling would reduce CO_2 production by altering the bacterial communities. We analyzed the fermentation quality, CO_2 production, and bacterial communities of fresh stylo and rice straw ensiled with pyroligneous acid.

MATERIALS AND METHODS

Silage Preparation

Stylo (*Stylosanthes guianensis*, CIAT 184) and rice (*Oryza sativa* L., Huahang 38) were planted without herbicide or fertilizer application in an experimental field of South China Agricultural University (23.24°N, 113.64°E, Guangzhou, China). Stylo (at the bloom stage) and rice straw (at the seed-harvesting stage) were harvested on August 08, 2020 and August 18, 2020, respectively. The two fresh materials were mixed and chopped to 1–2 cm by hand with a paper cutter, then treated with 1% or 2% pyroligneous acid based on fresh matter. Pyroligneous acid was obtained from blended wood waste and filtered through a 0.45-μm cellulose acetate membrane, similar to that reported by Zhang Y. et al. (2020). Approximately, 100 g of silage materials were packed and compressed manually into plastic-film bags (12 bags per treatment), sealed with a vacuum sealer, and stored indoors at 27–32°C. Silage samples from three bags were randomly collected after 3, 7, 14, and 30 days of fermentation, and the fermentation, gas production, and bacterial community parameters were determined.

Determination of Gas Production and CO_2 Concentration

The silage bag volume was measured in a 5000-mL beaker in a constant 25°C water bath. Gas production was then calculated using the difference in the volumes before and after silage (Cai et al., 1997). One microliter of the gas sample was collected with a microsyringe and injected into a gas chromatograph (Shimadzu GC-20A) to determine the CO_2 concentration. The CO_2 was separated on a molecular Sieve 5A and Porapak N column, with an oven temperature of 60°C for 5.5 min. The temperatures of the injector and detector were held at 100°C and 170°C, respectively. The quantitation limit of CO_2 in the gas chromatograph is 0.1% (v/v) using this method.

Fermentation Characteristics Analysis

The methods used to analyze the fermentation characteristics were similar to those used in our previous studies (Wang et al., 2018; He et al., 2020). Briefly, 20 g (including raw material and silage) of stylo and rice straw were taken randomly, soaked in 180 mL of sterile 0.9% saline for ∼15 min, and serially diluted from 10^{-1} to 10^{-6} on a clean bench. Lactic acid bacteria (LAB) and coliform bacteria were cultured and estimated using deMan-Rogosa-Sharpe agar and violet red bile agar at 30°C for 2 days. Yeast and mold were cultured and determined on Rose-Bengal agar for 2 days at 28°C. The 20 g of each silage sample were homogenized with 180 mL of distilled water for 18 h at 4°C, then filtered through four layers of cheesecloth and filter paper. A glass-electrode pH meter was used to immediately measure the pH of this filtrate. Organic acid contents were determined using high-performance liquid chromatography as described by Wang et al. (2018). The dry matter content was measured immediately after drying the samples at 65℃ using an electric dryer equipped with an air blower. The ammonia-N content was determined using a phenol-hypochlorite assay.

Bacterial Community Sequencing Analysis

The total bacterial DNA was extracted from the silage samples using a DNA kit (Omega Biotek, Norcross, GA,

United States) following the manufacturer's instructions and using specific steps as reported by Bai et al. (2020). The V3-V4 regions of the 16S rDNA were amplified using the primers, 341F: CCTACGGGNGGCWGCAG and 806R: GGACTACHVGGGTATCTAAT, and PCR was conducted using a 50-μL reaction mixture consisting of 1.5 μL of 5 μM of each primer, 1 μL KOD polymerase, 5 μL 10 × KOD buffer, 100 ng template DNA, and 5 μL of 2.5 mM dNTPs per the procedures of He et al. (2020). After purification and quantification, an Illumina HiSeq 2500 Sequencing System (Illumina, Inc., San Diego, CA, United States) was used for the PCR sequencing, and the raw sequences were analyzed as described by Wang et al. (2018). The bioinformatic data were examined via the free online platform at http://www.omicshare.com/tools by GENE *DENOVO*, and the QIIME bioinformatic pipeline[1] and principal coordinate analysis (PCoA) were used to calculate the α-diversity and β-diversity, respectively. The relative abundances of different bacterial communities at the phylum and genus levels were analyzed. The sequencing data were deposited in the Sequence Read Archive (SRA) under the accession number PRJNA735102.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 software, and the threshold for statistical significance was $P < 0.05$. All microbial count data were $\log_{10}$-transformed, and all figures were constructed using Adobe Illustrator CS 6.0.

RESULTS

Fermentation Properties of Stylo and Rice Straw Silage During Ensiling

Tables 1, **2** show the fermentation parameter dynamics of the stylo and rice straw silage. The lactic acid, acetic acid, and propionic acid contents increased, and the weight loss; pH; numbers of coliform bacteria, yeast, and mold and ammonia-N content decreased during ensiling (all $P < 0.05$).

Bacterial Community Dynamics During Ensiling

In the stylo silage treatments, PCoA1 and PCoA2 accounted for 28.6% and 50.7% of the total variance; in the rice straw silage treatments, PCoA1 and PCoA2 accounted for 13.8% and 16.3% of the total variance, respectively (**Figure 1**). **Figure 2** shows the relative bacterial community abundances at the phylum and genus levels. Proteobacteria and Firmicutes were the dominant phyla in both the rice straw and stylo silage, and their relative abundances decreased during ensiling. The abundance of *Lactobacillus* in the silage after pyroligneous acid treatment was higher than that in the control silage (**Figures 2**, **3**). Adding 1% pyroligneous acid increased the relative abundances of *Leuconostoc* on days 3 and 7 in the stylo silage, and adding 2% pyroligneous acid increased the relative abundances of *Leuconostoc* and *Lactococcus* in the rice straw silage on days 7 and 14, respectively (**Figures 2**, **3**). In the stylo silage, the relative abundance of *Novosphingobium* spp. increased, and the relative abundances of *Enterobacter* and *Kosakonia* decreased after pyroligneous acid treatment (**Figures 2**, **3**). Serine, arginine, nitrogen, glycine, proline, and threonine metabolism decreased after adding pyroligneous acid (**Figure 4**). Thus, adding pyroligneous acid may reduce potential pathogens on forage surfaces during ensiling (**Figure 5**). Adding pyroligneous acid also enhanced fermentation and reduced hydrocarbon degradation.

Gas and CO_2 Production During Ensiling

Gas was produced in accordance with CO_2 production and was drastically increased in both silages during week 1 (**Figure 6**). CO_2 production reached its maximum on day 14 (178 mL) in the naturally fermented stylo and on day 7 (317 mL) in the rice straw silage without additives owing to the different plant species and microorganisms in the two materials. Adding pyroligneous acid decreased the gas and CO_2 production in both silages. Adding pyroligneous acid to the laboratory silos reduced the CO_2 content of 100 g of stylo silage by 66 mL and of 100 g of rice straw silage by 84 mL.

The gas and CO_2 production increased substantially in the early stages of ensiling. Pyroligneous acid treatment increased the relative abundances of *Leuconostoc* spp. and decreased the pH and relative abundances of *Lactococcus* spp. After 14 days of ensiling without adding pyroligneous acid, the relative abundances of *Lactococcus* spp. decreased in the stylo silage. Adding 1% pyroligneous acid to the silage on days 14 and 30 increased and enhanced the relative abundances of *Clostridium* spp. (**Figures 2**, **3**). *Lachnoclostridium* was active on day 7 but was weakened during ensiling. The relative abundance of *Lachnoclostridium* also decreased after pyroligneous acid treatment. The relative abundance of *Prevotella* in the rice straw silage was higher on days 7 and 14 than on days 3 and 30 (**Figure 3**). Pyroligneous acid treatment decreased the relative abundances of *Selenomonas*, *Enterobacter*, *Prevotella,* and *Citrobacter*. Adding pyroligneous acid to the stylo silage increased the relative abundance of *Methylobacterium*.

DISCUSSION

Fermentation Properties of Stylo and Rice Straw Silage During Ensiling

Silage, a traditional method of preserving fresh forage, is very common and is used in ruminant production worldwide (Araújo et al., 2020). During ensiling, epiphytic microorganisms (mostly LAB) start fermentation under anaerobic conditions and produce lactic acid, causing the pH to decrease, inhibiting harmful microorganisms, and ultimately preserving the moist forage (Weinberg and Muck, 1996). Forages such as stylo and rice straw are difficult to directly ensile owing to their low water-soluble carbohydrate content and high abundance of undesirable microorganisms (Wang C. et al., 2019; He et al., 2020). Without pyroligneous acid, the fermentation quality of the stylo and rice straw silage was low because of the relatively high pH.

[1]https://qiime.org

TABLE 1 | Fermentation characteristics of stylo ensiled with or without pyroligneous acid (PA).

		Ensiling time (d)					SEM	Significance		
		3	7	14	30	Mean		T	A	T×A
Weight loss (%)	CK	0.39^{a}	0.83^{a}	1.40^{a}	2.71^{a}	1.33^{a}	0.761	<0.01	<0.01	<0.01
	1% PA	0.18^{b}	0.40^{b}	0.79^{b}	1.88^{b}	0.81^{b}				
	2% PA	0.15^{b}	0.34^{b}	0.62^{b}	1.32^{C}	0.68^{C}				
	Mean	0.25^{D}	0.54^{C}	0.93^{b}	1.97^{a}					
DM (%)	CK	30.3	27.9^{b}	28.0^{b}	27.0^{b}	28.3^{b}	1.15	<0.01	<0.01	0.06
	1% PA	30.6	28.9^{a}	29.5^{a}	29.3^{a}	29.3^{a}				
	2% PA	30.4	29.1^{a}	28.6^{AB}	28.9^{a}	29.6^{a}				
	Mean	30.4^{a}	28.6^{b}	28.7^{b}	28.4^{b}					
pH	CK	6.43^{a}	5.70^{a}	5.69^{a}	5.49^{a}	5.83^{a}	0.520	<0.01	<0.01	0.026
	1% PA	5.77^{b}	5.04^{b}	4.75^{b}	4.89^{b}	5.11^{b}				
	2% PA	5.43^{C}	4.97^{b}	4.84^{b}	4.71^{b}	4.99^{C}				
	Mean	5.87^{a}	5.24^{b}	5.10^{C}	5.03^{C}					
Lactic acid (% DM)	CK	0.93	0.80^{b}	0.65^{C}	0.71^{b}	0.77^{C}	0.16	0.012	<0.01	<0.01
	1% PA	1.00	1.05^{a}	0.96^{b}	0.82^{b}	0.96^{b}				
	2% PA	1.05	1.02^{a}	1.10^{a}	1.08^{a}	1.06^{a}				
	Mean	0.99^{a}	0.96^{AB}	0.90^{bC}	0.87^{C}					
Acetic acid (% DM)	CK	0.44^{b}	0.69^{b}	0.77	0.95	0.71^{b}	0.208	0.067	<0.01	<0.01
	1% PA	1.05^{a}	1.08^{a}	0.92	0.74	0.95^{a}				
	2% PA	1.13^{a}	1.07^{a}	0.89	0.82	0.98^{a}				
	Mean	0.87^{AB}	0.95^{a}	0.86^{AB}	0.84^{b}					
Propionic acid (% DM)	CK	ND	ND	ND	ND	ND	–	–	–	–
	1% PA	ND	ND	ND	ND	ND				
	2% PA	ND	ND	ND	ND	ND				
	Mean	ND	ND	ND	ND					
Butyric acid (% DM)	CK	ND	0.41	0.49	1.16	0.68	0.392	<0.01	–	–
	1% PA	ND	ND	ND	ND	ND				
	2% PA	ND	ND	ND	ND	ND				
	Mean	ND	0.41^{b}	0.49^{b}	1.16^{a}					
Lactic acid bacteria (Log_{10} cfu/g FM)	CK	7.90^{a}	7.81	7.48^{b}	7.20^{b}	7.60^{a}	0.696	<0.01	<0.01	<0.01
	1% PA	6.80^{b}	7.65	8.11^{a}	7.29^{b}	7.46^{a}				
	2% PA	5.55^{C}	7.56	7.87^{AB}	7.98^{a}	7.24^{b}				
	Mean	6.75^{C}	7.67^{a}	7.82^{a}	7.49^{b}					
Coliform bacteria (Log_{10} cfu/g FM)	CK	7.79^{a}	7.52^{a}	6.94^{a}	<3.00	7.42^{a}	1.624	0.022	<0.01	0.443
	1% PA	5.43^{b}	4.90^{b}	3.50^{b}	<3.00	4.61^{b}				
	2% PA	<3.00	<3.00	<3.00	<3.00	<3.00				
	Mean	6.85^{a}	6.21^{AB}	5.56^{b}	<3.00					
Yeasts and moulds (Log_{10} cfu/g FM)	CK	<2.00	<2.00	<2.00	<2.00	<2.00	–	–	–	–
	1%PA	<2.00	<2.00	<2.00	<2.00	<2.00				
	2% PA	<2.00	<2.00	<2.00	<2.00	<2.00				
	Mean	<2.00	<2.00	<2.00	<2.00					
Ammonia-N (% TN)	CK	6.59^{a}	8.89^{a}	11.4^{a}	14.3^{a}	10.3^{a}	4.33	<0.01	<0.01	<0.01
	1% PA	1.33^{b}	1.89^{b}	3.43^{b}	4.89^{b}	2.89^{b}				
	2% PA	1.01^{b}	1.25^{C}	1.82^{C}	3.00^{b}	1.77^{C}				
	Mean	2.98^{D}	4.01^{C}	5.53^{b}	7.41^{a}					

DM, dry matter; FM, fresh matter; TN, total N; SEM, standard error of means; ND, not detected; T, time of ensiling; A, additives; T×A, interaction of ensiling time and additives. Means with different letters in the same column (a–d) or row (A–D) indicate a significant difference (P <0.05).

Organic acids, especially acetic acid, are the main components of pyroligneous acid (Zhang Y. et al., 2020), which may partly explain the lower pH ($P < 0.05$) and higher acetic acid content ($P < 0.05$) in the present study in the pyroligneous acid-treated silages compared with those in the control silage. Plant cell respiration and microorganismal activities lead to nutrient losses during ensiling, especially in the early stages, and pyroligneous acid can decrease these losses possibly by direct acidification, which inhibits plant cell respiration and microbial activities. Acetic acid can be used to improve the aerobic stability of silage

TABLE 2 | Fermentation characteristics of rice straw ensiled with or without pyroligneous acid (PA).

		Ensiling time (d)					SEM	Significance		
		3	**7**	**14**	**30**	**Mean**		**T**	**A**	**T×A**
Weight loss (%)	CK	0.51^{a}	1.08^{a}	1.67^{a}	2.75^{a}	1.50^{a}	0.844	<0.01	<0.01	0.374
	1% PA	0.29^{b}	0.79^{b}	1.30^{b}	2.44^{b}	1.20^{b}				
	2% PA	0.21^{b}	0.61^{C}	1.29^{b}	2.43^{b}	1.22^{b}				
	Mean	0.35^{D}	0.83^{C}	1.42^{b}	2.54^{a}					
DM (%)	CK	39.6	37.3	38.3	37.3^{b}	38.1	1.11	0.055	0.175	0.059
	1%PA	38.6	38.6	38.5	39.6^{a}	38.8				
	2% PA	39.0	37.8	38.9	37.4^{b}	38.3				
	Mean	39.1^{a}	37.9^{b}	38.6^{AB}	38.1^{b}					
pH	CK	5.28^{a}	524^{a}	5.22^{a}	4.82^{a}	5.14^{a}	0.217	<0.01	<0.01	<0.01
	1% PA	4.85^{b}	4.82^{b}	4.82^{b}	4.75^{AB}	4.81^{b}				
	2% PA	4.96^{b}	4.76^{C}	4.72^{b}	4.69^{b}	4.78^{b}				
	Mean	5.03^{a}	4.94^{b}	4.92^{b}	4.75^{C}					
Lactic acid (% DM)	CK	0.60^{b}	0.65^{b}	0.61^{b}	0.68	0.63^{b}	0.058	<0.01	<0.01	0.089
	1% PA	0.67^{a}	0.73^{a}	0.74^{a}	0.73	0.71^{a}				
	2% PA	0.64^{AB}	0.75^{a}	0.72^{a}	0.74	0.72^{a}				
	Mean	0.63^{C}	0.71^{AB}	0.69^{b}	0.72^{a}					
Acetic acid (% DM)	CK	0.28^{b}	0.44^{C}	0.58	0.81^{a}	0.53^{b}	0.126	<0.01	0.034	<0.01
	1% PA	0.47^{a}	0.49^{b}	0.55	0.59^{b}	0.52^{b}				
	2% PA	0.48^{a}	0.61^{a}	0.58	0.59^{b}	0.57^{a}				
	Mean	0.41^{D}	0.51^{C}	0.57^{b}	0.66^{a}					
Propionic acid (% DM)	CK	ND	0.18	0.34^{a}	0.52^{a}	0.35^{a}	0.150			
	1%PA	ND	ND	ND	0.06^{b}	0.06^{b}				
	2% PA	ND	ND	ND	0.03^{b}	0.03^{b}				
	Mean	ND	0.18^{b}	0.34^{a}	0.23^{b}					
Butyric acid (% DM)	CK	ND	ND	ND	0.01	0.01	0.002	<0.01	-	-
	1%PA	ND	ND	ND	ND	ND				
	2% PA	ND	ND	ND	ND	ND				
	Mean	ND	ND	ND	0.01					
Lactic acid bacteria (Log_{10} cfu/g FM)	CK	8.81	8.69	8.20	7.77	8.36	0.488	<0.01	0.193	0.947
	1%PA	8.85	8.70	8.20	7.94	8.42				
	2% PA	8.63	8.58	8.14	7.50	8.21				
	Mean	8.76^{a}	8.65^{a}	8.18^{b}	7.74^{C}					
Coliform bacteria (Log_{10} cfu/g FM)	CK	7.61^{a}	7.60^{a}	5.93^{a}	<3.00	7.05^{a}	0.818	<0.01	<0.01	0.314
	1% PA	6.72^{b}	6.61^{AB}	5.57^{b}	<3.00	6.30^{b}				
	2% PA	6.37^{b}	5.79^{b}	<3.00	<3.00	6.08^{b}				
	Mean	6.90^{a}	6.67^{a}	5.75^{b}	<3.00					
Yeasts (Log_{10} cfu/g FM)	CK	4.76	4.43	3.14	<2.00	4.11	0.690	<0.01	0.885	0.173
	1%PA	4.35	4.24	3.86	<2.00	4.05				
	2% PA	4.62	3.95	3.14	<2.00	4.00				
	Mean	4.58^{a}	4.21^{a}	3.38^{b}	<2.00					
Moulds (Log_{10} cfu/g FM)	CK	4.20	<2.00	<2.00	<2.00	4.20	0.373	-	-	-
	1%PA	<2.00	<2.00	<2.00	<2.00	<2.00				
	2% PA	<2.00	<2.00	<2.00	<2.00	<2.00				
	Mean	4.20	<2.00	<2.00	<2.00					
Ammonia-N (% TN)	CK	4.31^{a}	10.4^{a}	14.7^{a}	17.6^{a}	11.7^{a}	4.700	<0.01	<0.01	<0.01
	1% PA	3.42^{b}	5.26^{b}	7.62^{b}	8.59^{b}	6.22^{b}				
	2% PA	1.97^{C}	3.38^{C}	4.74^{C}	6.41^{C}	4.12^{C}				
	Mean	3.23^{D}	6.35^{C}	9.02^{b}	10.9^{a}					

DM, dry matter; FM, fresh matter; TN, total N; SEM, standard error of means; ND, not detected; T, time of ensiling; A, additives; T×A, interaction of ensiling time and additives. Means with different letters in the same column (a–d) or row (A–D) indicate a significant difference ($P < 0.05$).

(Zhang et al., 2018). Therefore, the addition of pyroligneous acid might be helpful to improves the aerobic stability of silage and further study is needed.

Wang Y. et al. (2019) reported that lactic acid produced by LAB fermentation decreased the pH in the early stage of ensiling. In the current study, the lactic acid content increased

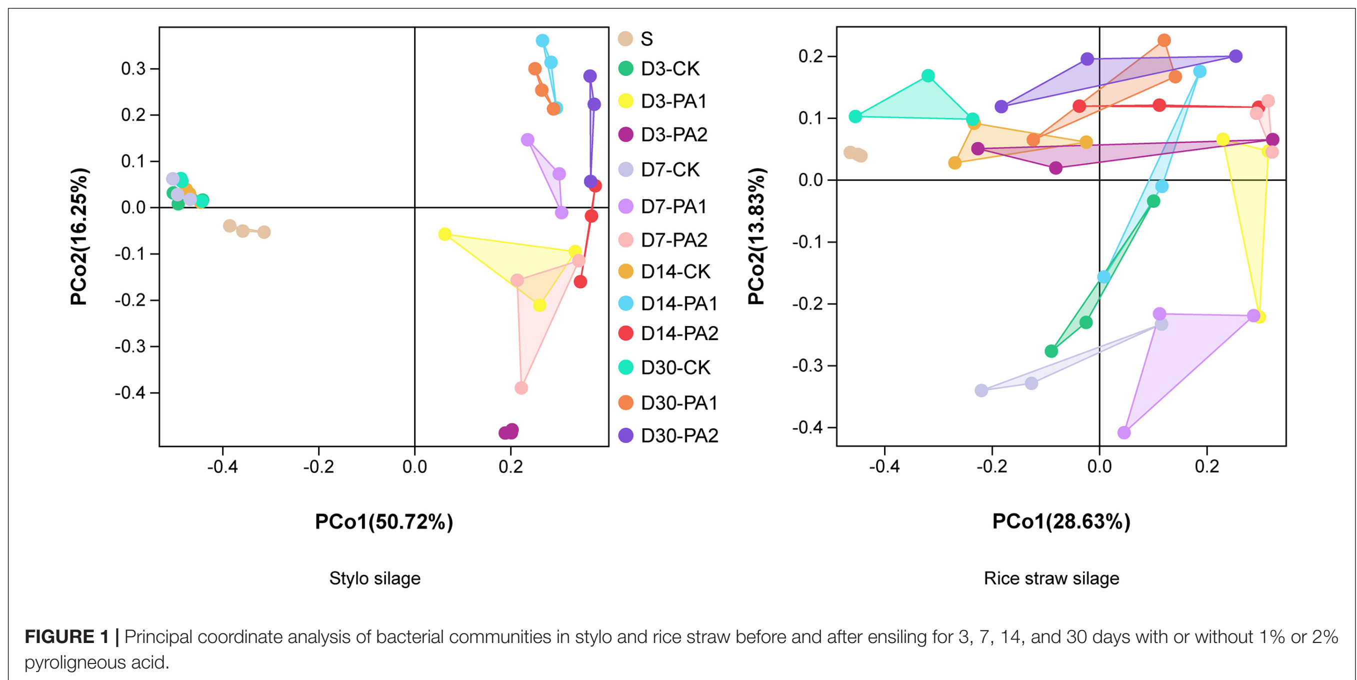

FIGURE 1 | Principal coordinate analysis of bacterial communities in stylo and rice straw before and after ensiling for 3, 7, 14, and 30 days with or without 1% or 2% pyroligneous acid.

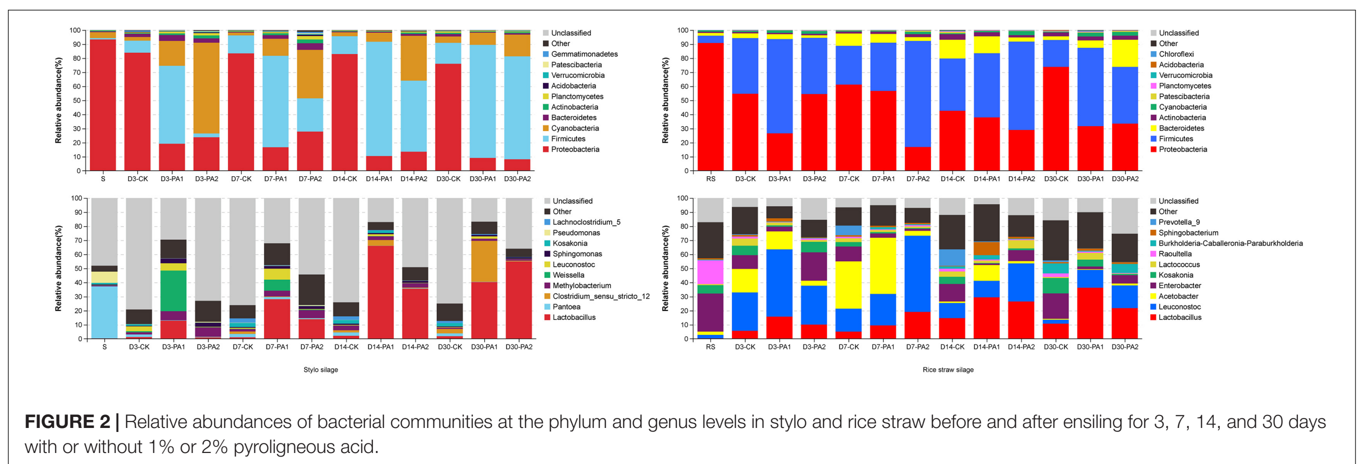

FIGURE 2 | Relative abundances of bacterial communities at the phylum and genus levels in stylo and rice straw before and after ensiling for 3, 7, 14, and 30 days with or without 1% or 2% pyroligneous acid.

in pyroligneous acid-treated silage. Butyric acid is an undesirable product in silage owing to the nutrient loss resulting from secondary fermentation caused by clostridial activity (McDonald et al., 1991). Pyroligneous acid treatment significantly decreased the butyric acid content ($P < 0.05$), possibly by inhibiting the activities of *Clostridium* spp. owing to the reduced pH after adding pyroligneous acid. Protein degradation results in non-protein-N and ammonia-N accumulation in silage, which have low utilization efficiency in ruminants, thus declining the silage quality (He et al., 2019).

Animal excretion negatively impacts the economy and ecology. Therefore, effective measures should be taken to reduce or prevent proteolysis in silage. The ammonia-N content is an important index of protein decomposition during ensiling (Pahlow et al., 2003) and is influenced by coliform bacterial activity. In this study, adding pyroligneous acid decreased the ammonia-N content ($P < 0.01$), which was consistent with the decreased coliform bacterial numbers. The mold numbers in the rice straw silage also decreased with pyroligneous acid treatment, similar to that reported by Jung (2007) and Suresh et al. (2019), who found that pyroligneous acid exerted growth-inhibiting effects on fungi such as *Aspergillus* spp.

Bacterial Community Dynamics During Ensiling

In this study, the improved fermentation quality was in accordance with the changes in the bacterial community during ensiling. The unweighted PCoA findings reflected the distinctions in the bacterial communities among treatments. Silages ensiled without additives were separated from the additive-treated silages, suggesting that pyroligneous acid affected the bacterial communities in the silage. *Lactobacillus* is the major LAB in silage and can grow rapidly and produce

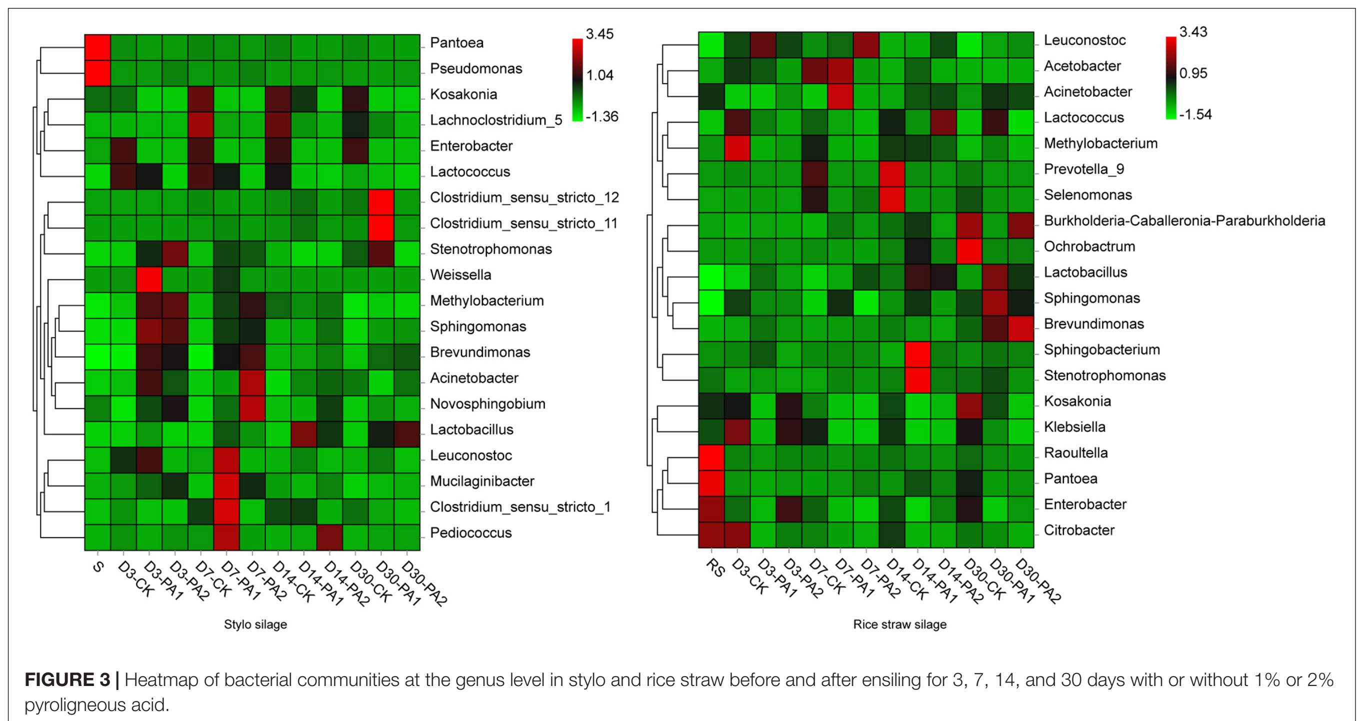

FIGURE 3 | Heatmap of bacterial communities at the genus level in stylo and rice straw before and after ensiling for 3, 7, 14, and 30 days with or without 1% or 2% pyroligneous acid.

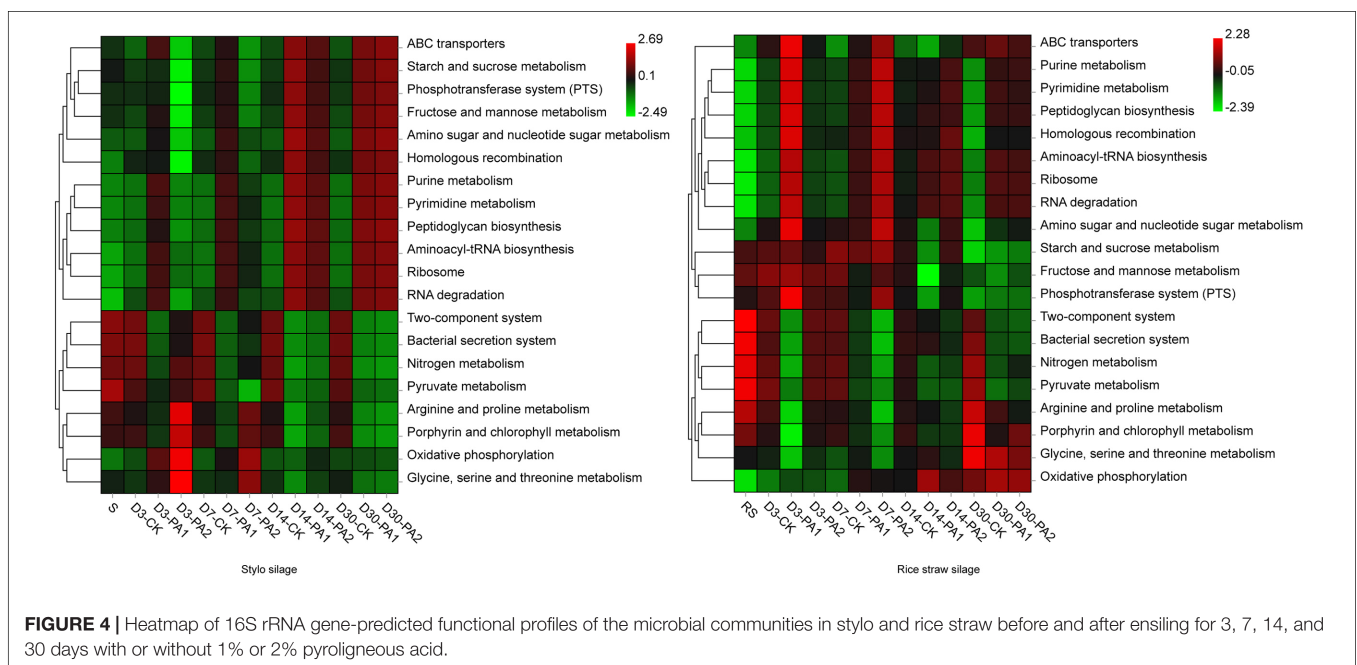

FIGURE 4 | Heatmap of 16S rRNA gene-predicted functional profiles of the microbial communities in stylo and rice straw before and after ensiling for 3, 7, 14, and 30 days with or without 1% or 2% pyroligneous acid.

lactic acid using water-soluble carbohydrates as substrates. *Lactobacillus* can also decrease the pH after oxygen is exhausted by plant cells and aerobic microorganisms in the early stage of ensiling. *Leuconostoc* and *Lactococcus* are main lactate-producing bacteria during ensiling and are usually used for fermentation in the early stage to effectively improve fermentation quality (Pahlow et al., 2003; Ni et al., 2018). *Novosphingobium*, a Gram-negative chemo-organotrophic bacterium, degrades various aromatic hydrocarbons. The increase in *Novosphingobium* spp. in the present study in the pyroligneous acid-treated stylo silage might have been due to the high aromatic hydrocarbon content in the pyroligneous acid (Zheng et al., 2020).

Enterobacter competes with LAB for oxygen and fermentation substrates and is an undesirable microorganism during ensiling; *Enterobacter* slows the decrease in the pH and increases protein degradation (Wang Y. et al., 2019). *Kosakonia* has characteristics similar to those of *Enterobacter* (Li et al., 2016), and its abundance in stylo silage was also reduced by

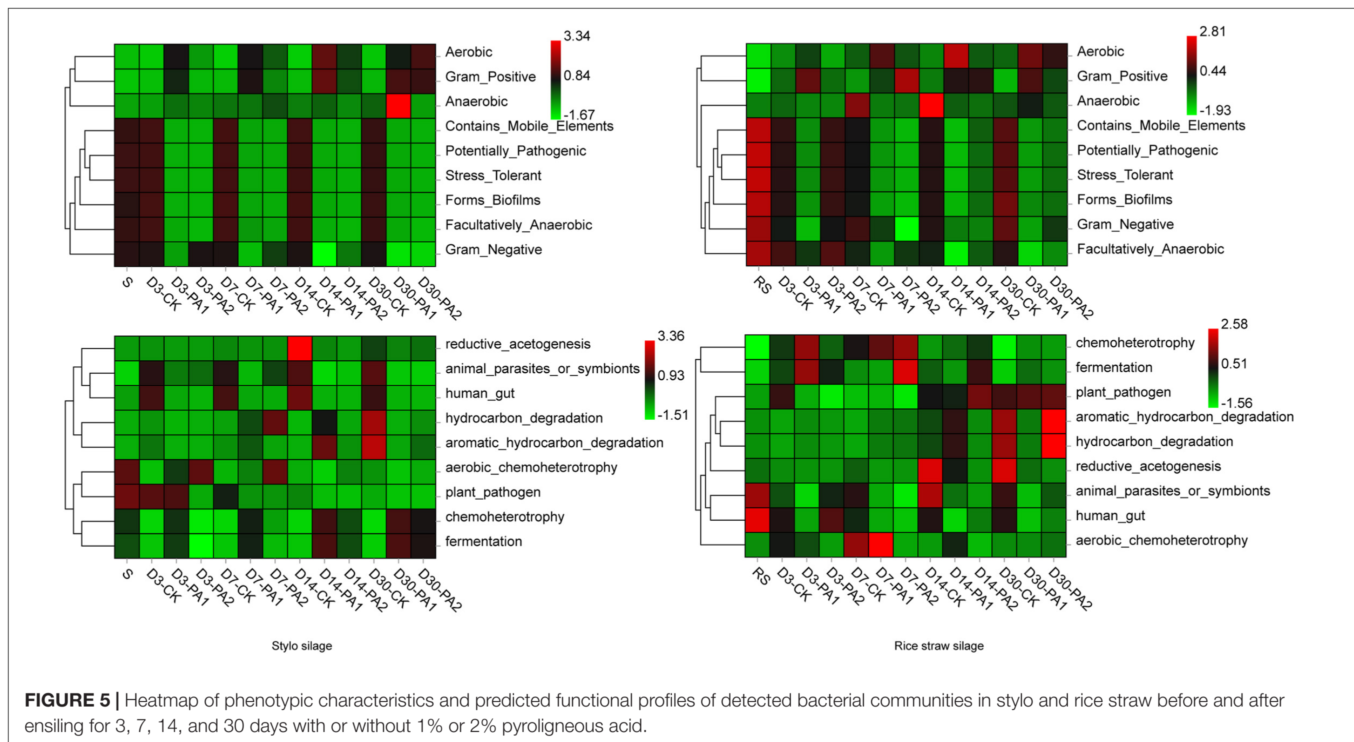

FIGURE 5 | Heatmap of phenotypic characteristics and predicted functional profiles of detected bacterial communities in stylo and rice straw before and after ensiling for 3, 7, 14, and 30 days with or without 1% or 2% pyroligneous acid.

pyroligneous acid treatment, possibly owing to the rapid decline in the pH that was inhibiting its growth. Notably, undesirable microorganisms such as *Enterobacter* can produce ammonia-N by fermenting amino acids. Dunière et al. (2013) reported that some amino acid decarboxylation may lead to accumulation of biogenic amines, which negatively affects animal health. Ensiling and adding pyroligneous acid improve fermentation quality owing to compounds such as organic acids, carbonyls, and phenolic derivatives—as well as the strong antimicrobial and antiviral activities of pyroligneous acid (Li et al., 2018; Suresh et al., 2019). Pyroligneous acid is reported to inhibit undesirable microorganisms, including *Escherichia*, *Enterobacter*, *Pseudomonas*, and *Listeria*, similar to the findings of the present study. Antibiotics are responsible for the present spread of multi-antibiotic-resistant bacteria, and many countries such as China ban the use of antibiotics in animal feed. Thus, as a natural antibacterial agent, pyroligneous acid may be a good alternative to conventional drugs in livestock farming.

Gas and CO_2 Production During Ensiling

In the early stage of silage fermentation, CO_2 and other gases are produced via respiration by plant cells and microorganisms, leading to gas accumulation. However, the gases produced from ensiling have attracted little attention although they may cause nutrient losses and the add to the greenhouse effect. Gas and CO_2 production drastically increased in both silages in week 1, which is similar to the findings of McEniry et al. (2011). *Lactobacillus casei* and *L. plantarum* in sorghum silage can reduce gas production; thus, rapid acidification during ensiling may decrease plant cell respiration and bacterial community changes (Cai et al., 1997). Gas production by both silages increased or decreased slowly after 7 days, possibly due to the environmental hypoxia and acidification inhibiting plant cell respiration and gas-producing bacteria. Silages are fermented for approximately, 30 days before being opened and fed to ruminants. In this study, adding pyroligneous acid reduced the CO_2 content and thus might be an effective method of reducing greenhouse gas emissions and mitigating climate change.

Gas and carbon dioxide production increased in the early stage of ensiling, which might be correlated with the relatively high abundances of *Leuconostoc* (3.41% and 27.2% in the stylo and rice straw silage on day 3, respectively). Adding pyroligneous acid increased the relative abundances of *Leuconostoc* spp., which was inconsistent with the reduction in CO_2 production in the pyroligneous acid-treated silages. In the present study, the pH and relative abundances of *Lactococcus* spp. decreased with pyroligneous acid treatment, possibly owing to the decreased CO_2 production in the pyroligneous acid-treated silages. Bacterial community alterations may explain the changes and reduction in CO_2 production during fermentation after adding pyroligneous acid. Zhai and Pérez-Díaz (2020) reported that *Leuconostocaceae* are the most important microorganisms that produce CO_2 during anaerobic fermentation. The relative abundances of *Leuconostoc* spp. increased, and CO_2 production decreased in pyroligneous acid-treated silages possibly because CO_2 production during fermentation is a complex process, and many other bacteria are involved. One study reported that increasing the initial pH from 6.0 to 6.8 significantly increased the CO_2 production rate of *Lactococcus* spp. (Andersen et al., 2005).

Clostridium spp. are considered a major CO_2 source in silage (Pahlow et al., 2003). The relative abundances of *Clostridium* spp. increased, possibly because some *Clostridium* spp. are

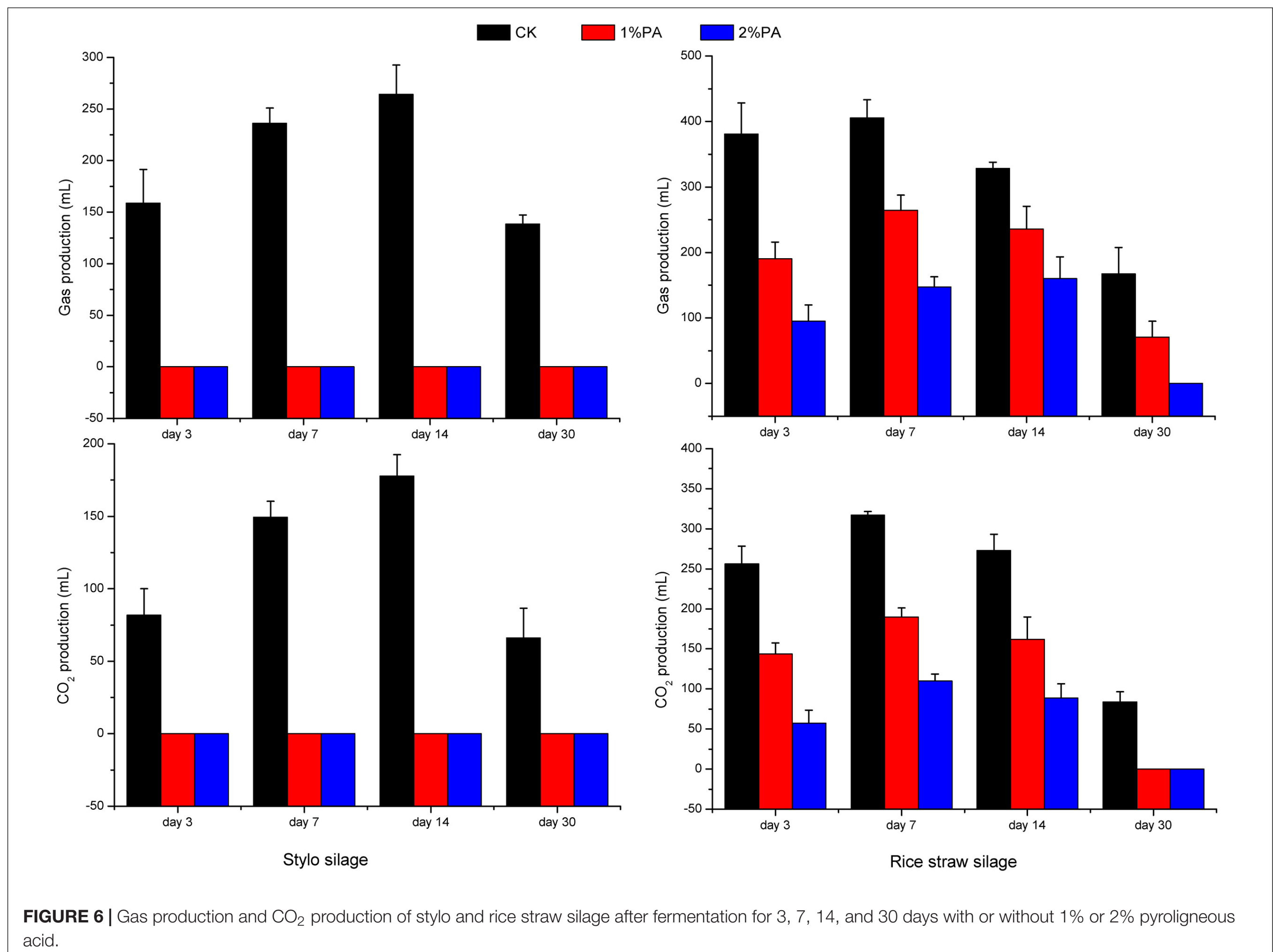

FIGURE 6 | Gas production and CO_2 production of stylo and rice straw silage after fermentation for 3, 7, 14, and 30 days with or without 1% or 2% pyroligneous acid.

autotrophic acetogenic bacteria that can produce important chemicals and fuels by using CO_2 (Zhang L. et al., 2020). *Lachnoclostridium*, a newly defined genus under the highly polyphyletic class *Clostridia*, showed a decreased relative abundance after pyroligneous acid treatment, consistent with that in CO_2 production. Furthermore, *Selenomonas*, *Enterobacter*, *Prevotella*, and *Citrobacter* may also be sources of CO_2 production during ensiling. Chen and Wolin (1977) presumed that *Selenomonas* spp. could ferment carbohydrates mainly to organic acids and CO_2; Converti and Perego (2002) reported that CO_2 is a major product of *Enterobacter aerogenes*; Emerson and Weimer (2017) reported that some *Prevotella* strains could produce CO_2 as the main product, and Lee et al. (2018) found that *Citrobacter amalonaticus* could produce CO_2 and H_2. In recent years, various approaches have been developed to reduce CO_2 emissions, among which, exploration of bacterial strains with CO_2 sequestration capacity might be effective. von Borzyskowski et al. (2018) reported that *Methylobacterium* could generate biomass from CO_2 using a heterologous Calvin-Benson-Bassham cycle. Okyay and Rodrigues (2015) considered that *Brevundimonas*, *Sphingobacterium*, *Pseudomonas*, and *Acinetobacter* strains can sequester CO_2. Furthermore, the abundance of *Stenotrophomonas*, which can fix CO_2, has been reported to increase in pyroligneous acid-treated silages (Okyay et al., 2016). Wang et al. (2020) added biochar and slag to paddy fields and observed a higher relative abundance of *Sphingomonas* and lower CO_2 emissions than those of the control fields and speculated that that *Sphingomonas* could reduce CO_2 emissions and sequestrate soil C. The increases in the relative abundances of *Methylobacterium*, *Brevundimonas*, *Sphingobacterium*, *Pseudomonas*, *Stenotrophomonas,* and *Acinetobacter* in pyroligneous acid-treated silage might also explain the reduced CO_2 production. Therefore, increasing the abundances of microorganisms that can sequester CO_2 may reduce greenhouse gas emissions and nutrient loss, and it might be possible to isolate such microorganisms from silage. Notably, CO_2 is used in many food products because high levels can inhibit the growth of some microorganisms. For example, CO_2 treatment was reported to reduce the abundances of detrimental bacteria, such as *Pseudomonas* and *Serratia*, in milk (Lo et al., 2016). Similarly, in this study, higher abundances of *Pseudomonas* were observed in silage without pyroligneous acid treatment. Thus, adding pyroligneous acid may reduce CO_2

production by changing the bacterial communities in rice straw and stylo silage.

CONCLUSION

Pyroligneous acid improved the fermentation quality of rice straw and stylo silage by increasing the lactic acid content and decreasing the weight losses, ammonia-N content, pH, butyric acid content, and coliform bacterial numbers. Additionally, pyroligneous acid increased the relative abundance of *Lactobacillus* and decreased that of undesirable bacteria such as *Enterobacter* and *Lachnoclostridium*. CO_2 production was reduced during ensiling, and pyroligneous acid treatment increased the relative abundances of CO_2-fixing genera. Given the immense production and demand for silage worldwide, application of pyroligneous acid may be an effective means of alleviating climate change caused by CO_2 emissions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA735102].

AUTHOR CONTRIBUTIONS

XG contributed to the investigation, software, data curation, formal analysis, and writing the original draft. PZ contributed to the investigation, methodology, isualization, and alidation. XZ contributed to the investigation, methodology, revision, and alidation. XC contributed to the conceptualization, funding acquisition, project administration, resources, and alidation. QZ contributed to the conceptualization, data curation, project administration, supervision, and validation. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Selective Thermotolerant Lactic Acid Bacteria Isolated From Fermented Juice of Epiphytic Lactic Acid Bacteria and Their Effects on Fermentation Quality of Stylo Silages

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The aim of the present study was to isolate and identify lactic acid bacteria (LAB) from fermented juice of tropical crops such as Napier grass, Ruzi grass, Purple guinea grass, Stylo legume, and Leucaena and their application to improve the quality of tropical crop silage. Fifteen strains of LAB were isolated. The LAB strains were Gram-positive and catalase-negative bacteria and could be divided into three groups, i.e., *Pediococcus pentosaceus*, *Lactiplantibacillus (para)plantarum*, and *Limosilactobacillus fermentum* according to the biochemical API 50CH test. Based on the analysis of 16S rRNA sequence, the strains isolated in the group *L. (para)plantarum* were distinguished. Two isolates (N3 and G4) were identified as *Lactiplantibacillus plantarum.* Three isolates (St1, St2, and St3) were identified as *L. paraplantarum.* In addition, the identification of other isolates was confirmed in the group *P. pentosaceus* (R1, R4, R5, R8, R11, and L1) and the group *L. fermentum* (N4, G6, G7, and N4). All selected strains were able to grow at 50°C. All LAB strains showed antimicrobial activity against *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931, *Pseudomonas aeruginosa* ATCC 27853, and *Bacillus cereus* ATCC 11778. Four selected LAB strains (St1, St3, N4, and R4) were tested for their capacity to successfully ensile Stylo legume (*Stylosanthes guianensis* CIAT184). Stylo silages treated with LAB were well preserved, the NH_3–N and butyric acid contents were lower, and the lactic acid content was higher than those in the control ($p < 0.05$). The acetic acid content was the highest in R4-treated silage among the treatments ($p < 0.05$). The crude protein (CP) content of St1-silage was significantly ($p < 0.05$)

higher than the others. The inoculation of thermotolerant LAB selected from fermented juice of epiphytic lactic acid bacteria (FJLB) was found to be highly instrumental to obtain well-preserved silage from the Stylo legume.

Keywords: forage crop, identification, inoculants, isolation, silage fermentation

INTRODUCTION

Ensiling is a widely used method of preserving moist forage for livestock in many countries. To achieve stable, nutritious silage, a rapid growth of lactic acid bacteria (LAB) is desired because LAB rapidly convert water-soluble carbohydrates (WSC) into lactic acid, thereby causing a rapid decrease in pH, which prevents, among others, excessive proliferation of clostridia (Ni et al., 2015). Naturally occurring epiphytic LAB populations on plant materials are generally heterofermentative and low in initial numbers (Cai, 1999; Eitan et al., 2006), causing a less successful fermentation as indicated by high pH and NH_3–N content in silage (Liu et al., 2011, 2012). Next to the aformentioned issues, high environmental temperatures may also complicate successful fermentation of forage (Chen et al., 2013; Li et al., 2019). The latter observation may be related to the inability of specific LAB to grow at high temperatures (Weinberg et al., 2001). Indeed, the temperature can reach values up to 50°C (Muck, 2010) when forage is ensiled under tropical conditions. Thus, the inoculation of forage with heat-tolerable LAB prior to ensiling may be instrumental to achieve a well-preserved silage. However, to the best of the author's knowledge, specific heat-tolerable epiphytic LAB are not yet identified and tested for their potential to successfully ensile tropical forages. In the current study, we isolated and identified LAB from fermented juice of epiphytic LAB (Bureenok et al., 2005) from various tropical forages. Fermented juice of epiphytic LAB (FJLB) contains multiple LAB strains (Wang et al., 2009) and is therefore considered a good source to screen for suitable LAB. Selected LAB were subsequently tested for their capacity to successfully ensile Stylo legume (*S. guianensis* CIAT184). Stylo was selected because of its practical relevance in the tropics.

MATERIALS AND METHODS

Preparation of FJLB and Isolation of LAB Strains

Forage-specific FJLB was prepared from fresh Napier grass (*Pennisetum purpureum*), Ruzi grass (*Brachiaria ruziziensis*), Purple guinea grass (*Panicum maximum* TD58), Stylo legume (*S. guianensis*), and Leucaena (*Leucaena leucocephala*) as described by Bureenok et al. (2005). Briefly, 25 g of each forage was macerated in 50 ml of distilled water in a blender. Then, the content of the blender was filtered over a double layer of sterilized cheese cloths into a glass bottle containing 1% glucose solution. The bottles were capped and stored under anaerobic conditions at 30°C for 3 days. Then, each forage-specific FJLB was spread on lactobacilli de Man, Rogosa, Sharpe (MRS) agar and incubated at 35°C for 48 h under anaerobic conditions. Thereafter, the predominant LAB colony was isolated and purified twice by streaking on MRS agar plates.

Morphological and Physiological Tests of the Selected Lactic Acid Bacterial Strains

Gram stain, morphology, catalase activity, and gas production from glucose were determined according to the methods for LAB identification as described by Kozaki et al. (1992). Growth at different pH values was observed in MRS broth (adjusting pH with 0.5 N HCl or NaOH) after incubation at 37°C. Growth at different temperatures was observed in MRS broth after incubation at 35°C and 45°C for 5 days. The turbidity of each tube was also noted as an indication of growth or no growth. Each treatment was tested with triplicate tubes. Growth curves for the isolates at 50°C were constructed by plotting the optical density at 600 nm against time. Carbohydrate fermentation was performed by API 50 CHI assay (BioMériux, Marcy-l'Étoile, France). LAB isolates were cultivated in 5 ml of MRS broth overnight at 30°C. The turbidity of the cultured broth was examined by the McFarland method. Cell suspension was transferred into each of the wells on the API 50 CH strips. All wells were coated with sterile liquid paraffin oil and incubated at 30°C. The results were read after 24 h and verified after 48 h. Fermentation of the carbohydrate medium was indicated by a yellow color, except for esculine (dark brown). Color reactions were scored against a chart provided by the manufacturer.

Identification of LAB Strains by 16S rRNA Sequence Analysis

The DNA of LAB isolates was extracted and purified using a Genomic DNA mini kit (Blood/culture cell) (Geneaid Biotech Ltd., Taiwan) according to the instructions of the manufacturer. Partial fragments of the 16S rRNA genes of each bacterial isolate were amplified using the forward primer 20F (5′-GAG TTT GAT CCT GGC YCA G-3′) and the reverse primer 1500R (5′-GTT ACC TTG TTA CGA CTT-3′) (Brosius et al., 1981). The polymerase chain reaction (PCR) mixtures contained the extracted DNA as a template, 2.0 mM $MgCl_2$, 0.2 mM dNTP, and 10 μl of 10X*Taq* buffer, 2.5 units of *Taq* polymerase, and the total volume was brought up to 100 μl. The PCR cycle of reactions consisted of an initial denaturation at 94°C for 3 min followed by 25 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and primer extension at 72°C for 1 min with a final extension at 72°C for 3 min. The amplicons of LAB were analyzed by means of gel electrophoresis using 0.8% (w/v) agarose and purified with a GenepHlow Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). The purified PCR products were sequenced by The Macrogen Laboratory (Seoul, South Korea). The resulting 16S rRNA gene sequence of the isolate was analyzed

and edited with the use of the Chromas 2.33 and BioEdit program (Hall, 1999). A comparative analysis of 16S rRNA gene sequences from the LAB isolates and all type strains related to the isolate was performed using CLUSTAL W version 1.83 (Thompson et al., 1994). The phylogenetic tree construction based on the 16S rRNA gene was performed using the neighbor-joining approach (Saitou and Nei, 1987) listed in the MEGA version 7 software (Kumar et al., 2016). The phylogenetic distances between the sequences were calculated according to Kimura's two-parameter model (Kimura, 1980). The robustness of individual branches of the tree was estimated by using bootstrap based on 1,000 replicates (Felsenstein, 1985). The 16S rRNA gene sequence similarities of the isolate were determined using the database of EZBioCloud[1] (Kim et al., 2012).

Inhibition Activity Determination of Lactic Acid Bacteria

The isolated LAB strains were inoculated in MRS broth and statically incubated at 30°C for 48 h. Cell-free supernatants were collected by centrifugation (10,000 × *g*, 4°C for 15 min) of LAB cultures and filtered through a 0.22-μm-diameter filter to remove residual cells. The agar well diffusion method (Li et al., 2015) was used to evaluate the antimicrobial activity of the selected LAB strains against the following indicator strains of bacteria: *Escherichia coli* ATCC 25922, *Shigella sonnei* ATCC 25931, *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative bacteria), and *Bacillus cereus* ATCC 11778 (Gram-positive bacteria). Cell-free supernatants (100 μl) were added into wells (7.80 mm in diameter) on nutrient agar plates inoculated with the indicator strains. All plates were incubated for 16–18 h at 30°C. The diameters of inhibition zones were recorded.

Preparation of the Experimental Silages

Stylo (*S. guianensis* CIAT184) was harvested 60 days after regrowth and chopped with a forage cutter to 2–4 cm and then sampled immediately to determine its macronutrient composition. Four selected LAB strains (*Lactiplantibacillus paraplantarum* St1, *L. paraplantarum* St3, *Limosilactobacillus fermentum* N4, and *Pediococcus pentosaceus* R4) were applied as silage additives. Next to the selected LAB strains, also forage-specific FJLB was prepared from fresh Stylo as previously described. Then, the Stylo was either or not inoculated with 1×10^5 CFU g^{-1} fresh forage of either forage-specific FJLB or one of the selected LAB strains. An equal volume of sterilized distilled water was added to Stylo that was not inoculated with LAB, and this treatment served as a control. Thereafter, ∼100 g fresh material of each experimental forage was tightly packed in oxygen impermeable plastic pouches (20.32 × 33 cm pouches, 120 μm thickness; M-PLASPACK, Bangkok, Thailand), and air was withdrawn from the plastic pouches by a vacuum sealer. Three pouches per treatment were prepared and stored at ambient temperature (37–42°C). After 45 days of ensiling, pouches were opened to assess ensiling characteristics and the macronutrient composition.

[1] http://eztaxon-e.ezbiocloud.net/

Chemical Analyses

The dry matter (DM) content of fresh Stylo and the experimental forages was determined after oven-drying at 60°C for 48 h. The nitrogen (N) contents were determined by the macro Kjeldahl method (AOAC, 1995). A factor of 6.25 was used to convert N into crude protein (CP). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to the method of Van Soest et al. (1991), and values are expressed inclusive of residual ash. Buffering capacity and WSC content were determined according to the method as described by Playne and McDonald (1966) and Dubois et al. (1956), respectively.

Lactic acid and volatile fatty acids (VFAs) in silage extracts were measured by HPLC (Aminex HPX-87H, 300 mm × 7.8 mm i.d.; column temperature, 40°C flow rate, 0.60 ml/min, Shimadzu Ltd., Kyoto, Japan). LAB in fresh Stylo legume and the experimental silages were enumerated on MRS agar, and plates were incubated at 35°C for 48 h. The NH_3–N content of the silage extract was determined using a steam distillation technique (Cai, 2004).

Statistical Analyses

Data were subjected to one-way analysis of variance (ANOVA); the differences between treatment means were compared using Tukey's *t*-test using SPSS for Windows version 16.0 SPSS (2007). Statistical Package for the Social Science. SPSS Inc., Chicago. United States. The level of statistical significance was declared at $p < 0.05$.

RESULTS

The Morphological and Physiological Properties of LAB Strains Isolated From FJLB

The isolation of bacteria using the MRS medium under anaerobic conditions allowed the identification of different LAB with similar or identical morphology from each FJLB. Based on the first step of the screening process, 15 strains were isolated from various forage-specific FJLBs: two strains from Napier grass (N1 and N4), five strains from Ruzi grass (R1, R4, R5, R8, and R11), four strains from Purple guinea grass (G3, G4, G6, and G7), three strains from Stylo legume (St1, St2, and St3), and one strain from Leucaena (L1). All strains were typified as Gram-positive and catalase-negative (**Table 1**). Among them, R1, R4, R5, R8, R11, and L1 were cocci; others were rod shaped. Based on the end products of glucose fermentation, strains N4, G3, G6, and G7 were classified as heterofermenters, while the remaining strains (N3, G4, St1, St2, St3, R1, R4, R5, R8, R11, and L1) were classified as homofermenters. Except for strain L1 at pH 8, all other strains grew well at various pH levels (3.5, 4.0, 4.5, and 8.0). Moreover, all strains grew well at 35 and 45°C. The ability to grow at 50°C was tested with all the strains (**Figure 1**) by measuring the density of cell populations in liquid culture over time, and it appeared that the strains St1, St2, St3, N3, and G4 exhibited slight growth, while the other strains grew well.

Based on the API 50CH results, the 15 isolates could be classified into three groups (**Supplementary Table 1**). Group

TABLE 1 | The characteristics of the selected lactic acid bacteria (LAB) isolates.

	Source of isolated strain of LAB														
	Napier grass		**Ruzi grass**					**Purple guinea grass**				**Stylo legume**			**Leucaena**
LAB strain	**N3**	**N4**	**R1**	**R4**	**R5**	**R8**	**R11**	**G3**	**G4**	**G6**	**G7**	**St1**	**St2**	**St3**	**L1**
Shape	Rod	Rod	Coccus	Coccus	Coccus	Coccus	Coccus	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Coccus
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	−	+	−	−	−	−	−	+	−	+	+	−	−	−	−
Fermentation type	Homo	Hetero	Homo	Homo	Homo	Homo	Homo	Hetero	Homo	Hetero	Hetero	Homo	Homo	Homo	Homo
Catalase activity	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth at pH															
3.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w
Growth at temperature															
35°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Identified as (16S rRNA)	*Lactiplantibacillus plantarum*	*Limosilactobacillus fermentum*	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*	*Limosilactobacillus fermentum*	*Lactiplantibacillus plantarum*	*Limosilactobacillus fermentum*	*Limosilactobacillus fermentum*	*Lactiplantibacillus paraplantarum*	*Lactiplantibacillus paraplantarum*	*Lactiplantibacillus paraplantarum*	*Pediococcus pentosaceus*

+, *positive*; −, *negative*; w, *weakly positive.*

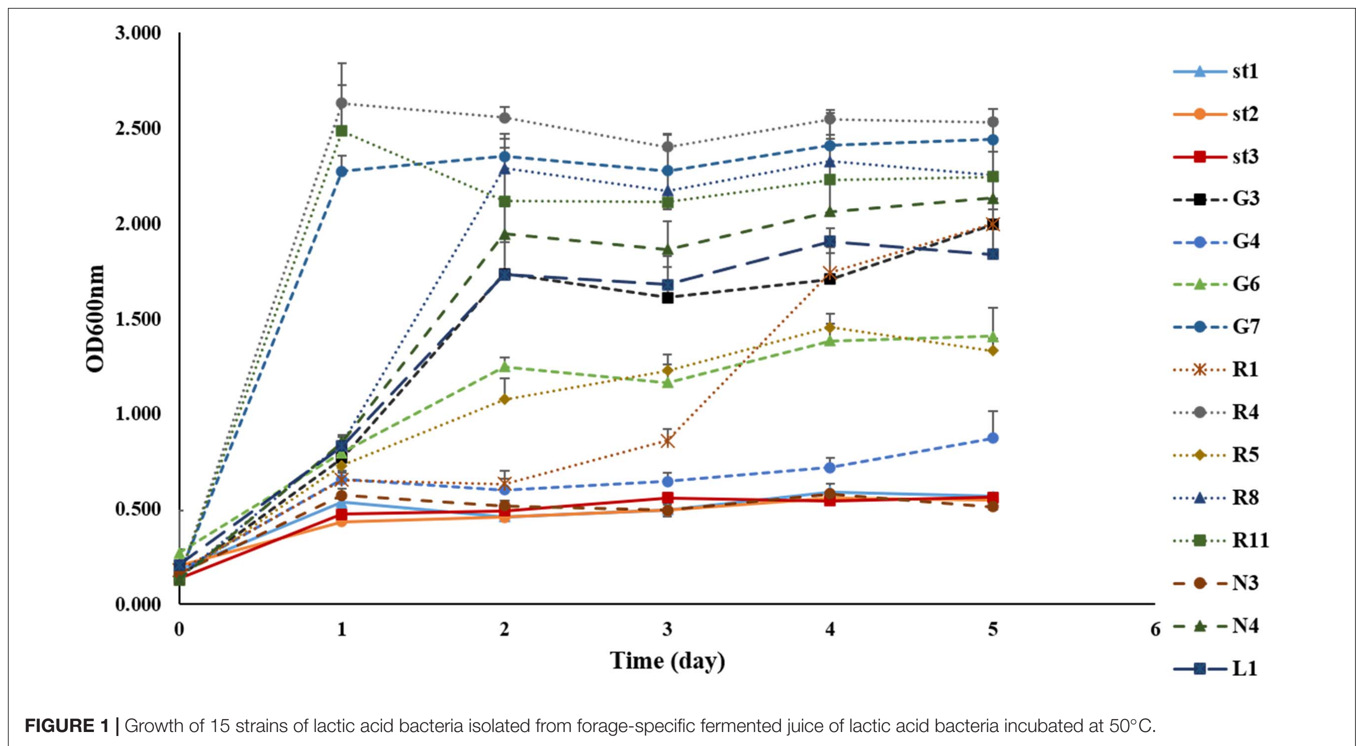

FIGURE 1 | Growth of 15 strains of lactic acid bacteria isolated from forage-specific fermented juice of lactic acid bacteria incubated at 50°C.

P. pentosaceus consisted of strains R1, R4, R5, R8, and R11 isolated from FJLB of Ruzi grass and strain L1 isolated from FJLB of Leucaena. LAB strains within this group were able to ferment xylose but not D-lactose, D-saccharose, and D-melibiose. Group *L. (para)plantarum* consisted of strain N3 (FJLB of Napier grass), strain G4 (FJLB of Purple guinea grass), and strains St1, St2, and St3 (FJLB of Stylo legume). The LAB strains within the group *L. (para)plantarum* were able to ferment α-methyl-D-mannopyranoside and D-lactose. Group *L. fermentum* contained the strains N4 (FJLB of Napier grass) and G3, G6, and G7 isolated from FJLB of Purple guinea grass. LAB within the group *L. fermentum* produced acid from D-raffinose but not *N*-acetyl glucosamine.

16S rRNA Gene Sequencing Analysis

In a phylogenetic tree based on 16S rRNA gene sequences, all 15 strains isolated from FJLB were divided into three groups similar to the API analysis (**Figure 2**). Six strains (R8, R5, R1, L1, R11, and R4) were grouped with *P. pentosaceus* on the phylogenetic tree with a bootstrap value of 100% and showing more than 99% similarity in their 16S rRNA gene sequences. Thus, these strains were identified as *P. pentosaceus*. Considering the phylogenetic positions observed, the type strain of *L. paraplantarum* DSM 10667^T was distinguished from *L. plantarum* CIP 103151^T. Strains N3 and G4 were close to the *L. plantarum* CIP 103151^T, with 100 and 99.91% similarity in their 16S rRNA gene sequences, respectively. Strains St1, St2, and St3 were categorized in the *L. paraplantarum* cluster and showed a similarity of 16S rRNA of 100% with *L. paraplantarum* DSM 10667^T. Four strains (N4, G3, G6, and G7) were most closely related to *L. fermentum* JCM 1173^T. Strains N4, G3, and G6 exhibited 100% 16S rRNA gene sequence pairwise similarities with the closely related species, *L. fermentum* JCM 1173^T. Only strain G7 showed 99.91% similarity of 16S rRNA gene sequences with the type strain *L. fermentum* JCM 1173^T.

Antibacterial Activity Against Pathogenic Bacteria

A total of 15 isolated LAB were tested for antagonistic activity against *E. coli*, *S. sonnei*, *P. aeruginosa*, and *B. cereus* by means of an agar diffusion test. Most of the strains showed antimicrobial activity against *E. coli* and *P. aeruginosa* (**Supplementary Table 2**). Except for strains G6 and G7, all other LAB strains showed zones of inhibition against *S. sonnei* and *B. cereus*.

Selected Indices of Fermentation and Chemical Composition of the Experimental Silages

The silage treated with St1 had a higher CP content compared with others (**Table 2**). N4-silages had lower WSC than the other silages. Lactic acid content was lower in the control silages ($p < 0.05$). The higher amount of acetic acid was higher in silage treated with *L. fermentum* N4. Except for the control silage, butyric acid could not be detected in all the silages treated with either FJLB or selected LAB. Stylo silage inoculated with all LAB strains had lower ($p < 0.05$) NH_3–N content than the control silage. Control silage pH was higher than the St1-treated silages ($p < 0.05$).

TABLE 2 | Chemical composition of fresh and ensiled Stylo, and selected indices of fermentation after 45 days of ensiling with or without additional LAB.

	Fresh Stylo	Control	Stylo ensiled with additional LAB					SEM	Sig.
			FJLB	LPL-1	LPL-3	LF N4	PP R4		
Chemical composition									
Dry matter (g kg^{-1} fresh)	280	275^{ab}	265^{ab}	279^{a}	258^{b}	266^{ab}	269^{ab}	1.44	0.017
Crude protein	102	90^{d}	101^{b}	109^{a}	98^{bc}	101^{b}	91^{cd}	0.69	<0.001
Neutral detergent fiber	689	673	680	686	649	659	693	6.99	0.487
Acid detergent fiber	499	510^{ab}	517^{a}	485^{ab}	472^{b}	497^{ab}	514^{ab}	3.70	0.026
Hemicellulose	190	163	163	201	177	162	179	7.32	0.631
Water-soluble carbohydrates	45.0	3.0^{b}	4.0^{ab}	4.8^{a}	4.5^{a}	1.4^{c}	4.1^{ab}	0.15	<0.001
Buffer capacity (meq kg^{-1} fresh)	250	na	na	na	na	na	na		
LAB (log CFU g^{-1} fresh)	4.88	6.70^{ab}	7.03^{a}	6.20^{b}	7.15^{a}	6.82^{a}	7.06^{a}	0.05	0.002
Lactic acid	na	33.91^{b}	55.54^{ab}	75.50^{a}	67.60^{ab}	86.75^{a}	81.72^{a}	2.93	0.002
TFa	na	52.91^{b}	64.34^{b}	85.16^{b}	84.43^{b}	148.47^{a}	89.18^{b}	4.12	<0.001
Profile of individual Fa (fraction of TFa)									
LA/TFa	na	0.62^{bc}	0.86^{a}	0.89^{a}	0.83^{ab}	0.58^{c}	0.91^{a}	0.18	<0.001
HAc/TFa	na	0.29^{ab}	0.14^{bc}	0.11^{bc}	0.17^{bc}	0.37^{a}	0.08^{c}	0.02	0.001
HBut/TFa	na	0.06^{a}	bdl	bdl	bdl	bdl	bdl	0.005	<0.001
HProp/TFa	na	0.02	0	0	0	0	0.04	0.003	0.093
NH_3–N (g kg^{-1} total N)	na	121.92^{a}	103.64^{ab}	70.12^{c}	80.64^{bc}	84.07^{bc}	88.76^{bc}	2.82	0.003
pH	5.64	4.71^{a}	4.40^{ab}	4.32^{b}	4.37^{ab}	4.41^{ab}	4.51^{ab}	0.03	0.047

Unless indicated otherwise, values are expressed as g kg^{-1} dry matter. CFU, colony-forming units; Con, Stylo ensiled without additional lactic acid bacteria; FJLB, fermented juice of epiphytic lactic acid bacteria; LPL-1, Lactiplantibacillus paraplantarum St1; LPL-3, Lactiplantibacillus paraplantarum St3; LF, Limosilactobacillus fermentum N4; PP, Pediococcus pentosaceus R4; TFa = total fermentation acids (i.e., lactic acid (LA) + acetic acid (HAc) + propionic acid (HProp) + butyric acid [HBut)]; na, not analyzed; bdl, below detection limit (zero value was used when data were statistically analyzed). Means with different superscripts within rows differ significantly ($p < 0.05$).

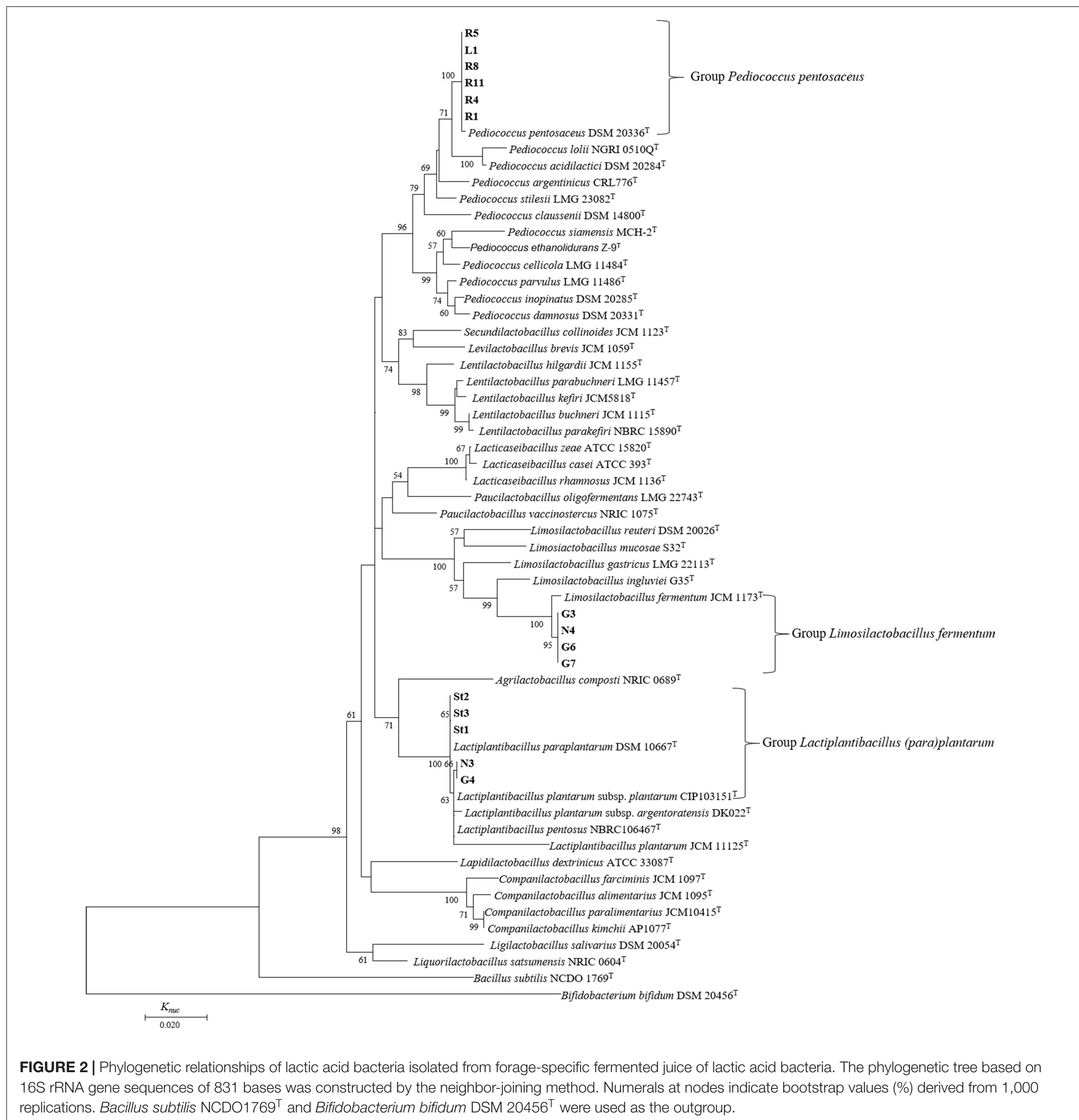

FIGURE 2 | Phylogenetic relationships of lactic acid bacteria isolated from forage-specific fermented juice of lactic acid bacteria. The phylogenetic tree based on 16S rRNA gene sequences of 831 bases was constructed by the neighbor-joining method. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications. *Bacillus subtilis* NCDO1769^{T} and *Bifidobacterium bifidum* DSM 20456^{T} were used as the outgroup.

DISCUSSION

LAB Strains Isolated From FJLB

Fermented juice of epiphytic LAB has been successfully used as an additive to ensile tropical grasses (Bureenok et al., 2005, 2011). In the current study, homolactic and heterolactic bacteria were isolated from the FJLB of different forage crops, i.e., Napier grass, Ruzi grass, Purple guinea grass, Stylo legume, and Leucaena. Homofermentative LAB produce two molecules of lactic acid from the fermentation of hexoses, whereas heterofermentative LAB produce one molecule of lactic acid, one molecule of other products (acetic acid, propionic acid, or ethanol), and CO_2 (Kung et al., 2003). In the case of facultative heterofermentation, LAB not only produce mainly lactic acid from hexose but also degrade pentose polymers, such as xylose, to lactic acid and acetic acid or ethanol (Oude Elferink et al., 2000). Based on the results of 16S rRNA analysis, the dominant LAB strains isolated from FJLB were identified as *L. plantarum*, *L. paraplantarum*, *L. fermentum*,

and *P. pentosaceus*. Khota et al. (2016) reported that the natural dominant strains of LAB species from Guinea grass and Napier grass were identified as *L. plantarum* and *Lacticaseibacillus casei* that could grow at lower pH and produce more lactic acid compared to the other isolates. Moreover, *L. plantarum* has been isolated from many kinds of grass such as king grass, vetch, tall fescue, and perennial ryegrass (Wang et al., 2017; Shah et al., 2018). The *Pediococcus* spp. have been observed as the prevalent species in forage plants or silages such as corn, Alfafa, Guinea, and Triticale grass (Cai et al., 1999; Kongsan et al., 2019; Soundharrajan et al., 2019). The predominance of LAB species that were found in the silage may be due to the prevalence of Mn^{2+} in plant materials (Daeschel et al., 1987; Boyaval, 1989). Epiphytic LAB such as *L. plantarum*, *L. fermentum*, and *P. pentosaceus* can accumulate Mn^{2+} from plants into their cells, thereby acting as a defense mechanism against oxygen toxicity (Daeschel et al., 1987; Kongsan et al., 2019). In this study, we screened thermotolerant LAB for developing a silage inoculant to be applied under tropical conditions. All LAB strains isolated from FJLB were able to grow at 50°C. Guo et al. (2020) reported that the *L. plantarum* strain isolated from the feces of dairy cows was able to grow at 50°C. Normally, the maximum temperature for optimum LAB growth and reproduction should not exceed 45°C (McDonald et al., 1991). Matsushita et al. (2016) reported that the genomic analysis of thermotolerant strains indicated a large number of mutations that are related to cell surface functions, ion and amino transporters, some transcription factor, and ROS (reactive oxygen species) in cells. There are many reports about LAB strains that have a limited capacity to adapt to high environmental temperatures and therefore have no positive effect on the process of fermentation during ensiling (Chen et al., 2013; Gulfam et al., 2017). Indeed, Guan et al. (2020) reported that LAB could not be detected after 60 days when corn was ensiled at 45°C instead of 30°C. Thus, it seems that thermotolerant LAB strains are potentially of interest to serve as an inoculant to achieve well-preserved silages in (sub)tropical regions. Furthermore, it was observed that most of the isolates in this study were able to inhibit the growth of pathogenic bacteria including *E. coli*, *S. sonnei*, *P. aeruginosa*, and *B. cereus*. Antimicrobial compounds produced by LAB were classified as organic acids, hydrogen peroxide, and bacteriocin-like compound (Heredia-Castro et al., 2015). Li et al. (2015) reported that 39 LAB strains isolated from corn stover silage had inhibitory effect against *Salmonella enterica* ATCC 43971^T, *E. coli* ATCC 11775^T, and *Micrococcus luteus* ATCC4698^T. The current LAB strains have the potential to inhibit the proliferation of undesirable and detrimental microorganisms, which also warrants the use of these LAB in silage making.

Fermentation and Chemical Composition of the Stylo Silages

Factors such as a low DM and WSC content and a high buffering capacity of material crop indicate poor conditions for lactic acid fermentation (Zhang et al., 2016). Moreover, initial LAB numbers in tropical forages are commonly too low for successful ensiling (Auerbach and Theobald, 2020). The DM content decreased by 0.28% in St1-silage after ensiling. The reduction of the CP content during the fermentation process was because of plant and microbial proteolytic processes in the ensiled material, which change the nitrogenous compounds in silages and result in an increase in soluble N and NH_3–N (Kung et al., 2018). The low pH values in all LAB-treated silages inhibited the growth of clostridia, which most likely prevented excessive CP loss (Tian et al., 2014). The ADF content of silages treated with St1 and St3 strains was reduced by 2.4–5.8% from the material crop, which is inconsistent with Liu et al. (2012). The decrease in the ADF content indicates a beneficial effect of the treatment in the improvement of the silage nutritive value and probably leads to an increase in silage digestibility in the rumen. It is generally accepted that well-preserved silages should contain pH values less than 4.5 and NH_3–N content not exceeding 100 g kg^{-1} total N (Kung et al., 2018). In this experiment, four strains of LAB, St1, St3, N4, and R4, were selected to serve as inoculants due to their ability to produce high lactic acid, to grow at a high temperature, to produce antimicrobial activity including the isolation source. In this study, the pH values in all LAB-treated silages were low enough to prevent protein degradation to NH_3–N. Compared with the homolactic bacteria, heterolactic *L. fermentum* N4 produced greater amounts of acetic acid, but lactic acid was still the predominant end product of fermentation. The current observation is in line with that of Lau and Liong (2014) who also reported that lactic acid was the main acid produced by *L. fermentum*. Despite the greater proportion of acetic acid in the silage treated with *L. fermentum* N4, both the low pH and NH_3–N values in this silage indicate that Stylo was successfully ensiled when *L. fermentum* N4 was used as an inoculant. The lactic acid content was lower in the control silages. Compared with the homolactic bacteria, heterolactic *L. fermentum* N4 also produced high lactic acid content. *L. fermentum* produced lactic acid in a more predominant amount than acetic acid in MRS broth (Lau and Liong, 2014). This may explain the high production of lactic acid in N4-silage. The higher amount of acetic acid was found when heterolactic *L. fermentum* N4 was added in the silages. Acetic acid is a main fermentation end product when silages are inoculated with heterolactic bacteria (e.g., *Lentilactobacillus buchneri*) with a content approximately 4% DM (Kleinschmit and Kung, 2006). Adding these strains would increase the acetic acid content to inhibit yeast, which could result in better aerobic stability of silages (Paradhipta et al., 2020). The intake of acetic acid (5% as DM basis) did not negatively affect the composition and sensory quality of milk (Daniel et al., 2013); hence, the level of acetic acid in this study will not affect the feed intake and animal performance. Propionic acid is an aerobic microbial inhibitor that can inhibit the activity of molds and yeasts (Chen et al., 2017). The strain of *L. fermentum* under application can increase the aerobic stability of the treated silage. The butyric acid content was higher ($p < 0.05$) in the control silages and not detected in all treated silages. This may be caused by LAB inoculation that reduced the growth of saccharolytic clostridia, which can ferment sugar, lactic acid, and acetic acid to butyric acid (Borreani et al., 2018). N4-silages had lower WSC than the other silages. This may be caused by a greater utilization of WSC to produce a high amount of lactic and acetic acid in this strain. However, low residual WSC content is an important factor for aerobic stability of silage because yeast and molds can utilize WSC resulting in

the rapid deterioration of silage after air exposure (Weinberg and Muck, 1996). The fermentation quality of the silages in the current study indicates that the microbial inoculants favorably affected the fermentation of Stylo legume.

CONCLUSION

Four selected strains, *L. paraplantarum* St1 and St3, *L. fermentum* N4, and *P. pentosaceus* R4, were shown to improve the fermentation quality and nutritive values of Stylo silage. The silage treated with St1 showed relatively high protein content than control and the other inoculants. The current results suggested that thermotolerant LAB strains isolated from FJLB could be used as a silage inoculant under tropical conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MW673709, https://www.ncbi.nlm.nih.gov/genbank/, MW673710, https://www.ncbi.nlm.nih.gov/genbank/, MW673711, https://www.ncbi.nlm.nih.gov/genbank/, MW673712, https://www.ncbi.nlm.nih.gov/genbank/, MW673713, https://www.ncbi.nlm.nih.gov/genbank/, MW673714, https://www.ncbi.nlm.nih.gov/genbank/, MW673715, https://www.ncbi.nlm.nih.gov/genbank/, MW673716, https://www.ncbi.nlm.nih.gov/genbank/, MW673717, https://www.ncbi.nlm.nih.gov/genbank/, MW673718, https://www.ncbi.nlm.nih.gov/genbank/, MW673719, https://www.ncbi.nlm.nih.gov/genbank/, MW673720, https://www.ncbi.nlm.nih.gov/genbank/, MW673721, https://www.ncbi.nlm.nih.gov/genbank/, MW673722, and https://www.ncbi.nlm.nih.gov/genbank/, MW673723.

AUTHOR CONTRIBUTIONS

SB, NP, and JS contributed to the conception and design of the study, and wrote sections of the manuscript. NP organized the LAB identifications. SB performed the silage experiment and the statistical analysis. SB and NP wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.673946/full#supplementary-material

Supplementary Table 1 | API 50 CH fermentation patterns of isolated LAB from each FJLB.

Supplementary Table 2 | Diameter of inhibition zones caused by the selected strains of LAB which were added to agar plates inoculated with either *Escherichia coli* ATCC 25922 (*E. coli*), Shigella sonnei ATCC 25931 (*S. sonnei*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), or *Bacillus cereus* ATCC 11778 (*B. cereus*). All values are expressed as mm, mean ± SD.

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METHODS
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A Multi-Sensor Mini-Bioreactor to Preselect Silage Inoculants by Tracking Metabolic Activity *in situ* During Fermentation

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The microbiome in silage may vary substantially from the onset to the completion of fermentation. Improved additives and inoculants are being developed to accelerate the ensiling process, to enhance fermentation quality, and to delay spoilage during feed-out. However, current methods for preselecting and characterizing these amendments are time-consuming and costly. Here, we have developed a multi-sensor mini-bioreactor (MSMB) to track microbial fermentation *in situ* and additionally presented a mathematical model for the optimal assessment among candidate inoculants based on the Bolza equation, a fundamental formula in optimal control theory. Three sensors [pH, CO_2, and ethanol (EtOH)] provided data for assessment, with four additional sensors (O_2, gas pressure, temperature, and atmospheric pressure) to monitor/control the fermentation environment. This advanced MSMB is demonstrated with an experimental method for evaluating three typical species of lactic acid bacteria (LAB), *Lentilactobacillus buchneri* (LB) alone, and LB mixed with *Lactiplantibacillus plantarum* (LBLP) or with *Enterococcus faecium* (LBEF), all cultured in De Man, Rogosa, and Sharpe (MRS) broth. The fermentation process was monitored *in situ* over 48 h with these candidate microbial strains using the MSMB. The experimental results combine acidification characteristics with production of CO_2 and EtOH, optimal assessment of the microbes, analysis of the metabolic sensitivity to pH, and partitioning of the contribution of each species to fermentation. These new data demonstrate that the MSMB associated with the novel rapid data-processing method may expedite development of microbial amendments for silage additives.

Keywords: lactic acid bacteria (LAB), multi-sensor mini-bioreactor (MSMB), fermentation, silage additive, metabolic sensitivity, pH, carbon dioxide (CO_2), ethanol (EtOH)

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INTRODUCTION

Silage is a major feedstuff for ruminant animal production worldwide. The biochemical production of silage relies on anaerobic lactic acid fermentation. Homofermentative lactic acid bacteria (LAB) ferment glucose to lactic acid as the primary by-product, whereas heterofermentative LAB ferment glucose to lactic acid, acetic acid, ethanol (EtOH), and carbon dioxide (CO_2)

(Muck, 2004; Kung et al., 2018a; Muck et al., 2018). Testing for heterofermentative fermentation generally involves gas phase sampling of CO_2 and EtOH (McEniry et al., 2011; Li et al., 2017; Kung et al., 2018b).

Though silage is a nutritious and palatable animal feed, its aerobic deterioration is inevitable when the silo is opened for feeding out, but spoilage can be reduced significantly using either biological or chemical additives (Ranjit and Kung, 2000; Borreani and Tabacco, 2010; Tabacco et al., 2011; Wilkinson and Davies, 2012). Commercial silage inoculants contain highly selected bacteria that speed up silage acidification during anaerobic fermentation (Queiroz et al., 2013; Muck et al., 2018). This typically involves strains of facultative heterofermentative bacteria such as *Lactiplantibacillus plantarum* (LP), and obligate heterofermentative bacteria such as *Lentilactobacillus buchneri* (LB) are used to enhance the aerobic stability of silage (Bolsen et al., 1996; Kung et al., 2018a; Muck et al., 2018). Rapid acidification significantly inhibits the growth of undesirable microorganisms and reduces fermentative loss, while acetic acid, the by-product of heterofermentation, suppresses the aerobic proliferation of spoilage-causing fungi and minimizes oxidative losses during feed-out (Pahlow et al., 2003; Muck, 2004; Kung et al., 2018b).

To continuously improve these biological silage additives requires selection of improved microbial strains from among the abundant species and strains of LAB that are present in natural populations (Muck et al., 2018). Selection is commonly performed during the natural ensiling process, which is time-consuming, laborious, and costly, requiring large numbers of *ex situ* samples and intensive laboratory analyses (Weinberg and Ashbell, 2003). To resolve these bottlenecks in research for silage inoculants, this study presents a multi-sensor-based screening method with three major objectives: (i) to present an alternative model for selecting microbes, (ii) to devise a multi-sensor mini-bioreactor (MSMB) for screening microbial strains during fermentation *in situ*, and (iii) to evaluate the capabilities of the advanced MSMB in selection among candidate inoculants.

MATERIALS AND METHODS

Microbial Selection and Optimal Fermentation

With the use of control system theory (Sargent, 2000), a LAB fermentation process $[X(t)]$ can be described as a set of state variables $[x_1(t) – x_3(t)]$ under microbial control u_m such that

$$\dot{X} = \begin{bmatrix} \dot{x}_1 \\ \dot{x}_2 \\ \dot{x}_3 \end{bmatrix} = F[(x_1(t), x_2(t), x_3(t), u_\mathrm{m}, t)] \quad \text{with } \mathrm{t}_0 \le \mathrm{t} \le \mathrm{t}_\mathrm{f} \tag{1}$$

where x_1, x_2, and x_3 refer to the accumulated productions of organic acids (lactic and acetic), CO_2, and EtOH, respectively, with respect to the initial time (t_0) and the final time (t_f) of fermentation. As the function of time (t), these state variables can be expressed as the outputs of triple integrators such that

$$\left.\begin{aligned} x_1(t) &= \tfrac{1}{\mathrm{pH}(t)} = \int_{t_0}^{t} [\mathrm{acid}]\,\mathrm{dt} \\ x_2(t) &= \int_{t_0}^{t} [\mathrm{CO_2}]\,\mathrm{dt} \\ x_3(t) &= \int_{t_0}^{t} [\mathrm{EtOH}]\,\mathrm{dt} \end{aligned}\right\} \tag{2}$$

where [acid] is the instantaneous production of organic acids (primary lactic and acetic), resulting from LAB fermentation. Similar representations apply to carbon dioxide [CO_2] and [EtOH]. According to optimal control theory (Sargent, 2000), an optimal/minimum cost function of $\hat{J}$ subject to Eq. 2 exists, and an optimal control ($\hat{U}_{m-best}$) fulfills

$$\begin{aligned} \hat{J}\left(\hat{u}_{\mathrm{m}-best}\right) &= \min.(x_1+x_2+x_3) \\ &= \min.(t_f-t_0)_{\mathrm{pH}\downarrow} + \min. \int_{t_0}^{\min.\ t_f} [\mathrm{CO_2}\ (t)]\mathrm{dt} \\ &+ \min. \int_{t_0}^{\min. t_f} [\mathrm{EtOH}\ (t)]\mathrm{dt} \end{aligned} \tag{3}$$

where $\min.(t_f - t_0)_{pH\downarrow}$ is the shortest time of acidification dynamics of Eq. 1, i.e., the optimal time to be determined by fermentation. The two integrations of $x_2(t)$ and $x_3(t)$ are related to minimum fermentative loss. Eq. 3 is a special case of the generalized problem of Bolza (Clarke, 1976; Sargent, 2000), which contains a global solution of the time-energy optimization subject to an optimal control function ($\hat{u}_{m-best}$). In this study, the optimal time-energy trajectories of fermentation were determined experimentally by selecting candidate microbes.

Multi-Sensor-Based Experimental System

The instrumental structure of the MSMB (**Figure 1**) contains seven different functional sensors (**Table 1**), with six enclosed in an air-tight chamber. According to Eq. 2, pH, CO_2, and EtOH are the three indicators of the LAB fermentation, each corresponding to an analog integrator. Additionally, micro-environmental parameters, i.e., O_2 concentration, ambient temperature (T_a), and gas pressure (P_{gas}) in the sealed chamber, were measured during the fermentation process. P_{gas} was measured relative to ambient air pressure (P_{air}), which was measured using a digital barometer placed on the outside of the sealed chamber (**Figure 1**). Throughout the test, P_{gas} remains positive ($P_{gas} > P_{air}$) due to accumulation of CO_2 and volatile EtOH. The P_{gas} measurement has two functions: (i) to determine the seal characteristics of the chamber before and during the experiment and (ii) to compensate the O_2 measurement since the optical O_2 sensor was calibrated under P_{air}.

The MSMB including the air-tight chamber (glass jar, 1.5 L) is schematically shown in **Figure 2**. A centrifuge tube (inner diameter 2.5 cm, height 12 cm) was filled with fermentation medium (orange color) for culturing the candidate LAB. The pH electrode was immersed in the medium, as the integrator to determine the acidification characteristics of the fermentation relative to each strain or species. The resulting gas from the

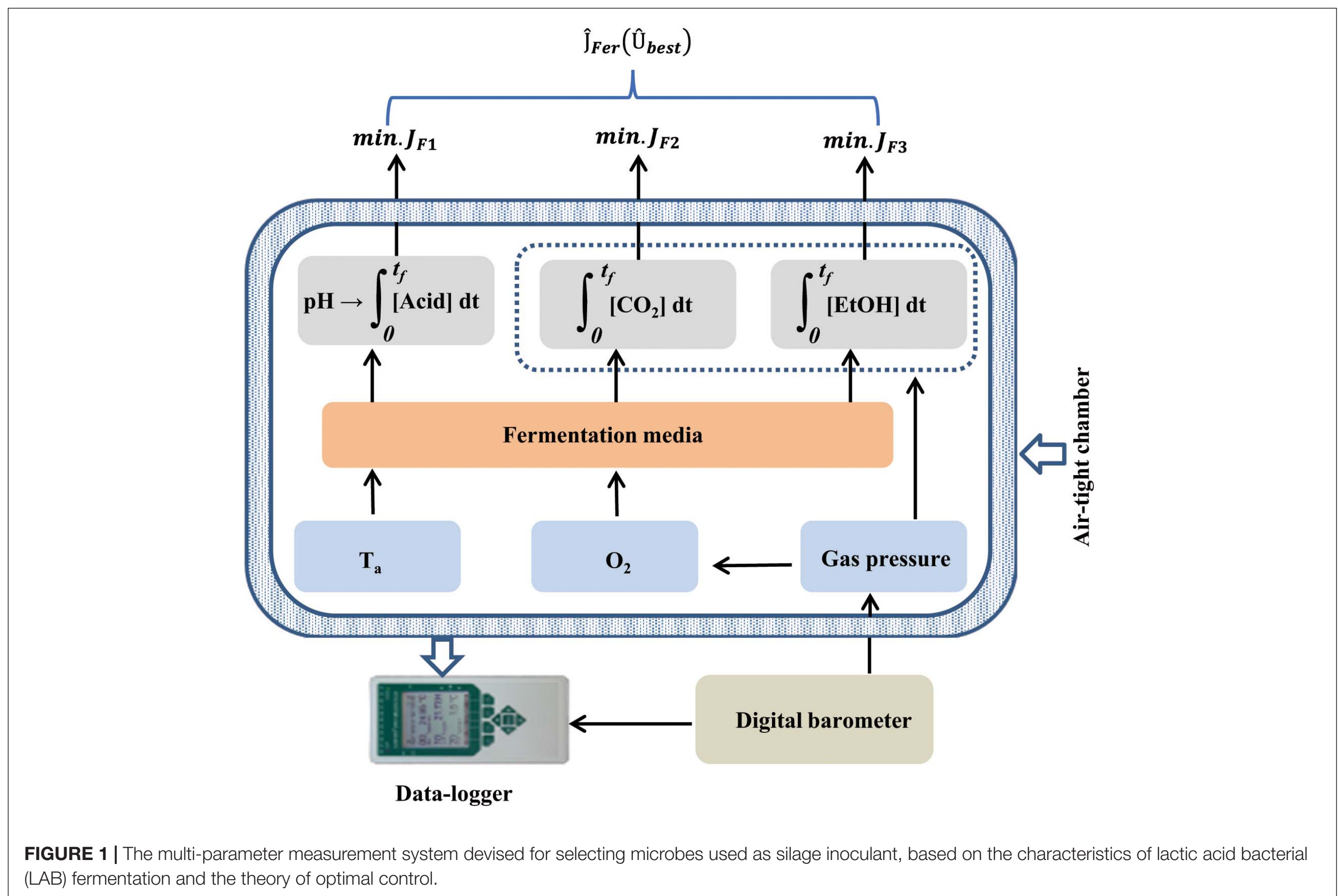


FIGURE 1 | The multi-parameter measurement system devised for selecting microbes used as silage inoculant, based on the characteristics of lactic acid bacterial (LAB) fermentation and the theory of optimal control.

TABLE 1 | Technical information of the pH, CO_2, ethanol (EtOH), O_2, temperature, and pressure sensors used.

Parameter	Sensor-type	Manufacturer	Range	Accuracy	Response(s)
pH	BlueLine 21	SI Analytic GmbH, Germany	2–13	±0.3	<20
Carbon dioxide	Prime 2	Clairair Ltd., United Kingdom	0–5% (vol.)	±3% of full scale	<60
Ethanol	TGS2610	Figaro USA, Inc.	500–10,000 (ppm)	±10% of final value in range	<300
Oxygen	OXROB10	Pyro Science GmbH, Germany	Gas phase: 0–100% (vol.)	Gas phase: ± 0.2%	Gas phase: < 7
			Dissolved oxygen:0–44 mg/L	Dissolved oxygen: ± 0.1 mg/L	Dissolved oxygen: < 15
Temperature	FTA15 NiCr-Ni ZA9020FS	Ahlborn Mess-und Regelungstechnik GmbH, Germany	−50 to 200°C	± 0.1°C	1.5
Atmospheric pressure	FDAD12SA	Ahlborn Mess-und Regelungstechnik GmbH, Germany	700–1,050(mbar)	± 0.5% of final value in range	<5
Gas pressure	FDA612SR	Ahlborn Mess-und Regelungstechnik GmbH, Germany	± 1,000(mbar)	±0.5% of final value in range	<5

fermentation diffuses out of the centrifuge tube through six holes (diameter 2 mm) in the wall of the upper tube. Because CO_2 is unreactive with EtOH, this allows the glass jar to act as a dual integrator for simultaneous collection of the CO_2 and the volatile EtOH from the fermentation process in the centrifuge tube. The O_2 sensor can be moved vertically to measure the O_2 concentration in the gas space or O_2 dissolved in the medium. To remove O_2 for anaerobic requirements, paired holes (diameter 3 mm) were perforated in the lid (**Figure 2B**) for purging with N_2. Three sets of the MSMB provided replication for simultaneous

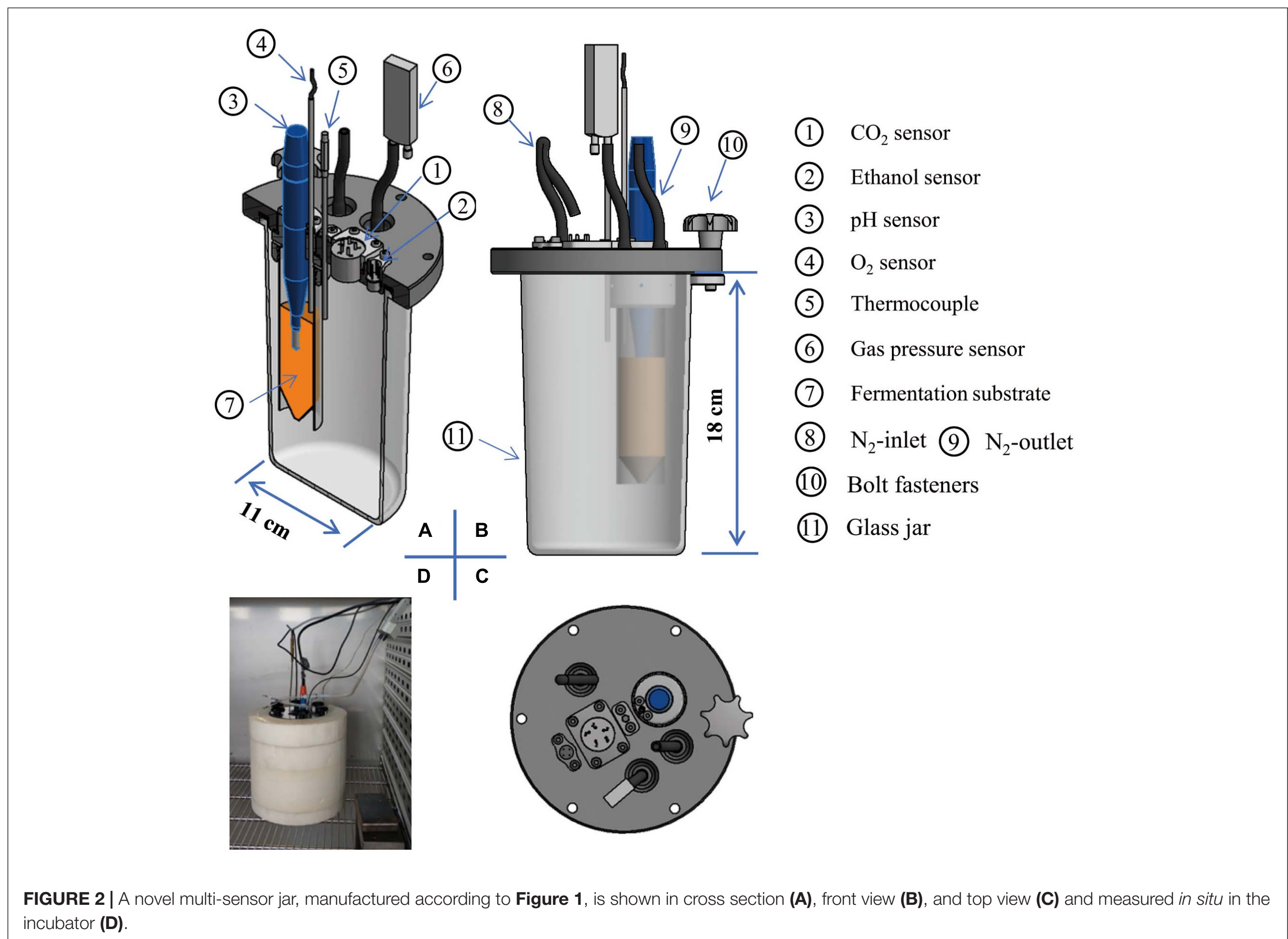


FIGURE 2 | A novel multi-sensor jar, manufactured according to **Figure 1**, is shown in cross section **(A)**, front view **(B)**, and top view **(C)** and measured *in situ* in the incubator **(D)**.

testing. Three data loggers were linked to (i) the thermocouples and pH electrodes (ALMEMO-2890-9, nine-channel, Ahlborn Mess-und Regelungstechnik GmbH, Ilmenau, Germany), (ii) CO_2 and EtOH sensors (own manufacture, eight-channel), and (iii) the O_2 sensor (four-channel, Pyro Science GmbH, Aachen, Germany), all sampled at 10-min intervals.

Sample Preparation

Strains of typical species of obligate heterofermentative bacteria, *Lt. buchneri* DSM 13573 (LB) and two typical species of facultative heterofermentative bacteria, *Lp. plantarum* DSM 3676, *Lp. plantarum* DSM 3677, and *Enterococcus faecium* NCIMB 11181 (EF), were chosen for the experiment. The two strains of *Lb. plantarum* were used as a mixture (1:1) in all experiments and designated as LP. *Lt. buchneri* DSM 13573 (LB) was used individually and mixed with *Lp. plantarum* DSM 3676/DSM 3677 (LBLP) or with *E. faecium* NCIMB 11181 (LBEF).

All candidate bacteria were prepared as lyophilizates by cultivating the strains on synthetic medium and harvesting the cells by centrifugation. The harvested biomass was lyophilized at −40°C for 2 days; and the lyophilizates were stored at −18°C. The cell density of the lyophilized LB was 1.6×10^{12} CFU/g. As 0.1 g of the lyophilized LB with 40 ml of De Man, Rogosa, and Sharpe (MRS) (**Table 2**) was cultured during the experiment, the resulting inoculum density of LB was 4×10^9 CFU/ml. For LBLP and LBEF, either *Lp. plantarum* DSM 3676/DSM 3677 or *E. faecium* NCIMB 11181 was added with 10% of the cell density of the LB (i.e., 4×10^8 CFU/ml). The cell density of the lyophilized LP was 1×10^{11} CFU/g, and that of the lyophilized EF was 3×10^{12} CFU/g. Thus, 0.160 g of the lyophilized LP and 0.005 g of the lyophilized EF were mixed with 0.1 g of the lyophilized LB. These microbial samples, in 40 ml of sterile MRS broth in each centrifuge tube (vol. 50 ml), were incubated inside the multi-sensor instrumented jar at 30°C for 48 h.

Chemical Analyses

All the fermented samples were frozen in the centrifuge tubes at −20°C immediately after incubation, prior to chemical analyses. The fermentation acids (lactic and acetic), EtOH, and propanediol were determined using high-performance liquid chromatography (HPLC; KNAUER Azura, Wissenschaftliche Geräte GmbH, Berlin, Germany), coupled with integrated UV and refractive index (RI) detectors as described by Shan et al. (2019).

TABLE 2 | Composition of the De Man, Rogosa, and Sharpe (MRS) broth.

Substance	Con.	Substance	Con.	Substance	Con.
Peptone	10 g/l	Yeast extract	4 g/l	Dipotassium phosphate	2 g/l
Glucose	20 g/l	Sodium acetate	5 g/l	Ammonium citrate	2 g/l
Beef extract	8 g/l	Polysorbate 80	1 g/l	Magnesium sulfate ($MgSO_4$)	0.2 g/l

MRS, De Man, Rogosa, and Sharpe.

Signal Processing

Normalized Productions of $\overline{CO_2}$ and $\overline{EtOH}$

To compare the relative rate of increase of the CO_2 or EtOH in the fermentation process, the normalized productions of carbon dioxide ($\overline{CO_2}$) and $\overline{EtOH}$ were calculated, respectively, as

$$\overline{CO_2(t)} = \frac{\int_0^t [CO_2]\, dt}{\int_0^{t_f} [CO_2]\, dt} \qquad 0 \leq t \leq t_f \tag{4}$$

and

$$\overline{EtOH(t)} = \frac{\int_0^t [EtOH]\, dt}{\int_0^{t_f} [EtOH]\, dt} \qquad 0 \leq t \leq t_f \tag{5}$$

where both $\overline{CO_2}$ and $\overline{EtOH}$ vary from 0 to 1 as functions of t.

Temporal Rate of ΔCO_2 and $\Delta EtOH$

This differential variable may reflect the dynamics of metabolic activity of microorganisms. As sequences of discrete-time data, the differentials of CO_2 and EtOH were calculated over time as

$$\left.\begin{aligned} \triangle CO_2 &= [CO_2\,(t_i)] - [CO_2\,(t_{i-1})] \\ \triangle EtOH &= [EtOH\,(t_i)] - [EtOH\,(t_{i-1})] \\ t_0 &= 0,\ t_n = t_f,\ i = 1,\ 2,\ \ldots .n \end{aligned}\right\} \tag{6}$$

Signal Decomposition

Signal decomposition, a function of smart instruments, is often used to partition a mixed source signal into its constitutive pure components for various engineering problems (Li et al., 2013; Shan et al., 2019). In this study, the fermentation characteristics relative to different strains or combinations are regarded as mathematical curves in functional space. Three time courses of pH (i.e., pH_{LB}, pH_{LBLP}, and pH_{LBEF}) are directly tracked *in situ* from the experiment. Through data decomposition, pH_{LP} and pH_{EF} can also be obtained, such that

$$\left.\begin{aligned} pH_{LP}\,(t) &= pH_0 + \left(pH_{LBLP}\,(t) - pH_{LB}\,(t)\right) \\ pH_{EF}\,(t) &= pH_0 + \left(pH_{LBEF}\,(t) - pH_{LB}\,(t)\right) \end{aligned}\right\} \tag{7}$$

where pH_0 is the initial value of each substrate.

Statistical Analysis

The experimental data were analyzed using IBM SPSS v25.0 (IBM Co., Armonk, NY, United States). Linear regression, curve fitting, and fitting errors were evaluated using coefficient of determination (R^2) and root mean square error (RMSE). Two-way analysis of variance (ANOVA) was conducted for effects of the experimental scheme (two air environments, i.e., anaerobic and aerobic), treatment (three types, i.e., LB, LBEF, and LBLP), and the interactions of the chemical compositions for the final-data processing. One-way ANOVA was used to evaluate the statistical significance among anaerobic and aerobic environments.

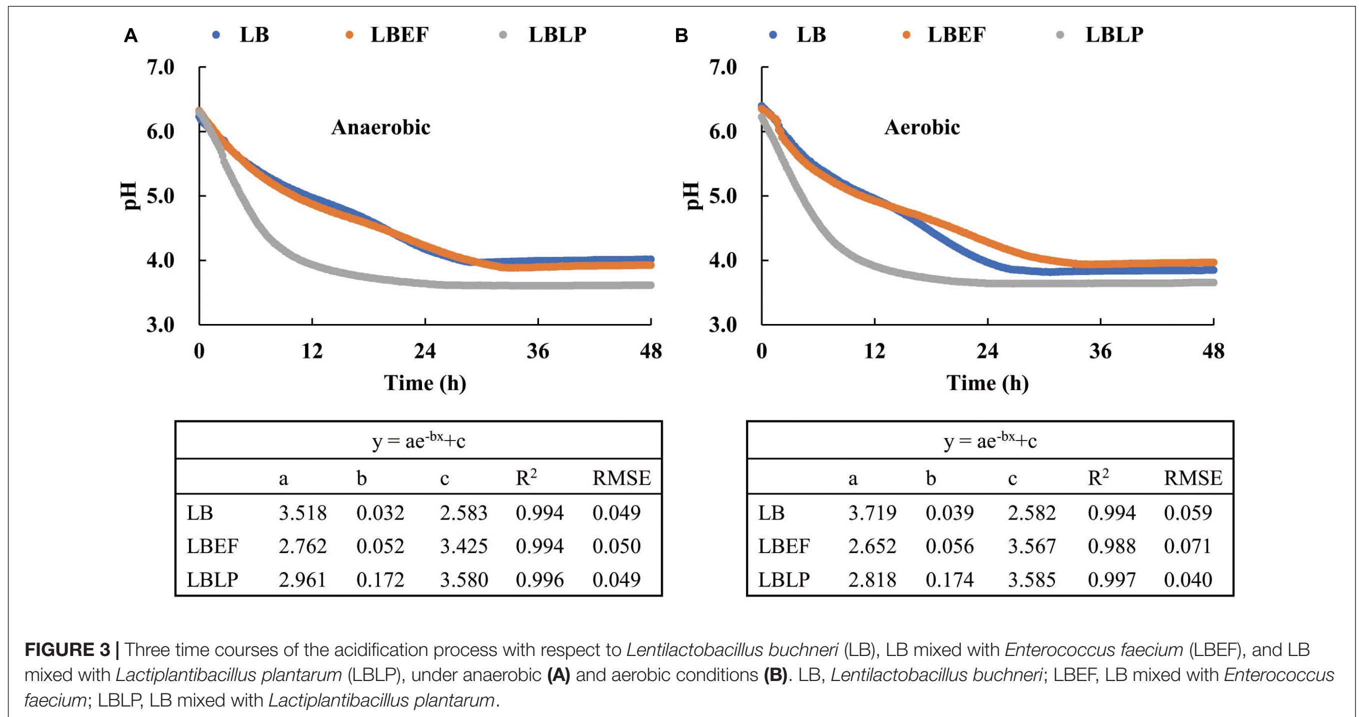
RESULTS

Acidification Characteristics

Two sets of time courses of pH (**Figure 3**), each with respect to the mean of three replicates, were recorded *in situ* from the fermentation process in the MSMB, the first set (**Figure 3A**) from anaerobic fermentation and the second set (**Figure 3B**) from aerobic fermentation. The patterns observed were similar. **Figure 4** shows the time courses of O_2 concentration over the experiment with two parts, i.e., as liquid phase dissolved in the MRS and as gaseous phase distributed in the glass jars. When comparing the anaerobic fermentation (**Figure 4A**) and aerobic one (**Figure 4B**), the amounts of gaseous oxygen in these jars remained two constant levels throughout the experiment, i.e., around 0.2 vol. % of O_2 concentration in the anaerobic jars and 20 vol. % of O_2 concentration in the aerobic jars. The O_2 dissolved in the MRS in both anaerobic and aerobic jars varied with similar trends in the initial 5 h, i.e., declined from 0.157 to 0.138 mg/L, and then reached a plateau. This and the chemical analyses from aerobic and anaerobic fermentations at the end of the experiment (**Table 3**) demonstrate that the oxygen in the MSMB had minimal impact on the respiratory metabolism of the microbes tested.

In general, all time courses of pH (**Figures 3A,B**), i.e., the acidification characteristics, were well described by exponential regressions, in both anaerobic and aerobic fermentations. The steepest decline was observed from the LBLP (pH_{LBLP} = 5, t = 4.5 h, anaerobic), evidently faster than that of LBEF (pH_{LBEF} = 5, t = 10 h, anaerobic) or that of the LB (pH_{LB} = 5, t = 11.5 h, anaerobic). Additionally, the pH_{LBLP} had the lowest values (pH_{LBLP} = 3.61 or 3.65) in both anaerobic and aerobic fermentations, which agreed with the finding that the contents of lactic acid in the fermented MRS of LBLP are slightly higher than those of LB and LBEF (**Table 3**).

The final time (t_f) was determined as pH $\leq$ 4 over six consecutive measurements (i.e., 1 h). The t_f of the pH_{LBLP} was only 12 h, whereas the t_f for both pH_{LBEF} and pH_{LB} was more than double at 28 h. Therefore, for these microbial samples in the same experimental conditions, the acidification characteristics of the LBLP had the lowest t_f (i.e., the fastest process). Therefore, the resulting time course of pH_{LBLP} is time optimal, but this only fulfilled by one of the two necessary criteria for a global



(A) $y = ae^{-bx}+c$	a	b	c	R^2	RMSE
LB	3.518	0.032	2.583	0.994	0.049
LBEF	2.762	0.052	3.425	0.994	0.050
LBLP	2.961	0.172	3.580	0.996	0.049

(B) $y = ae^{-bx}+c$	a	b	c	R^2	RMSE
LB	3.719	0.039	2.582	0.994	0.059
LBEF	2.652	0.056	3.567	0.988	0.071
LBLP	2.818	0.174	3.585	0.997	0.040

FIGURE 3 | Three time courses of the acidification process with respect to *Lentilactobacillus buchneri* (LB), LB mixed with *Enterococcus faecium* (LBEF), and LB mixed with *Lactiplantibacillus plantarum* (LBLP), under anaerobic **(A)** and aerobic conditions **(B)**. LB, *Lentilactobacillus buchneri*; LBEF, LB mixed with *Enterococcus faecium*; LBLP, LB mixed with *Lactiplantibacillus plantarum*.

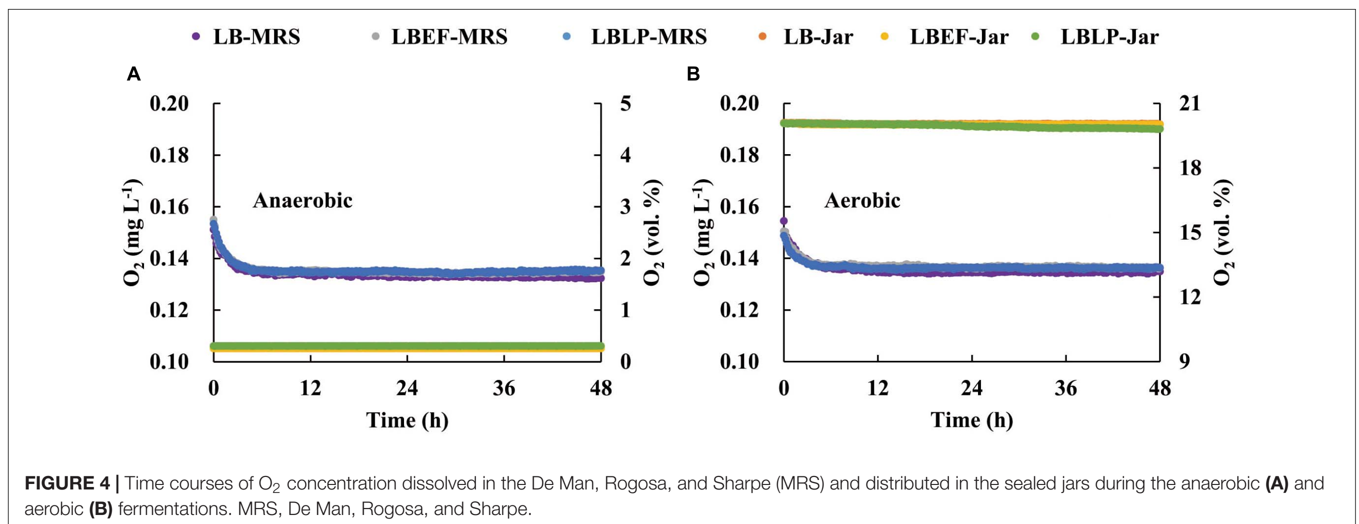


FIGURE 4 | Time courses of O_2 concentration dissolved in the De Man, Rogosa, and Sharpe (MRS) and distributed in the sealed jars during the anaerobic **(A)** and aerobic **(B)** fermentations. MRS, De Man, Rogosa, and Sharpe.

TABLE 3 | Final chemical analyses of the three types of samples.

Variable	Anaerobic			Aerobic			SEM	Significance of effects		
	LB	LBEF	LBLP	LB	LBEF	LBLP		A	T	A × T
pH	4.06^{a}	4.07^{a}	3.61^{b}	4.10^{A}	3.96^{A}	3.65^{B}	0.048	NS	**	NS
Lactic acid (g/l)	13.56	12.23	16.44	12.77	13.52	15.33	1.238	NS	NS	NS
Acetic acid (g/l)	5.74	5.27	4.82	5.78	6.08	4.66	0.420	NS	NS	NS
Ethanol (g/l)	3.36^{a}	1.81^{b}	1.08^{c}	3.47^{A}	1.95^{B}	1.00^{C}	0.133	NS	**	NS
Propanediol (g/l)	0.327^{a}	0.317^{a}	0.253^{b}	0.397^{A}	0.453^{A}	0.207^{B}	0.037	NS	**	NS

SEM, standard error of the mean; A, air environment (anaerobic or aerobic); T, treatment (LB, LBEF, or LBLP).
LB, Lentilactobacillus buchneri; LBEF, LB mixed with Enterococcus faecium; LBLP, LB mixed with Lactiplantibacillus plantarum; NS, not significant.
$^{**}p < 0.01$.
$^{a-c,\ A-C}$Means with different superscripts within the same air environment differ significantly ($p < 0.05$).

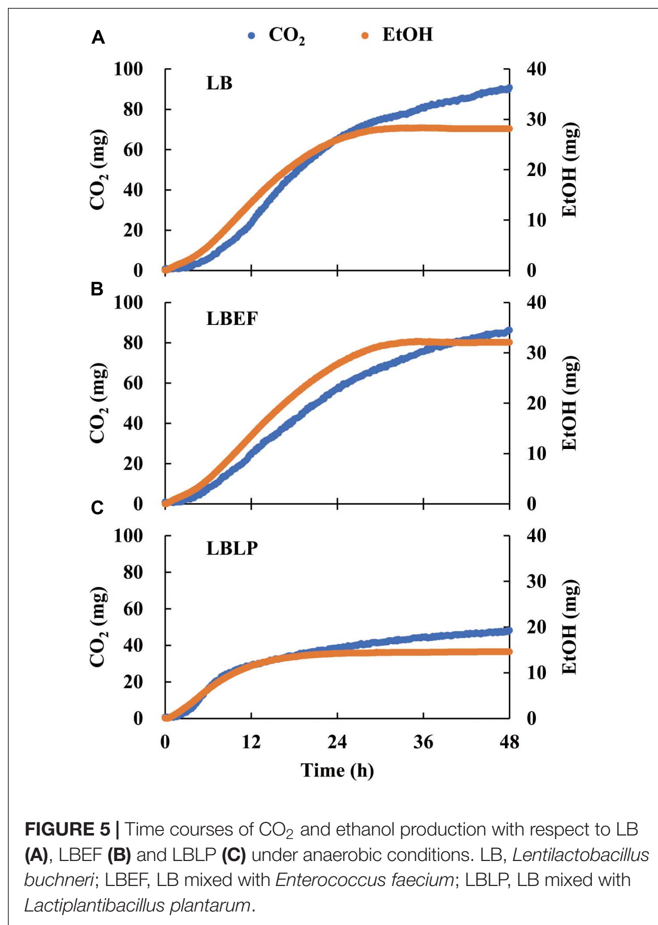


FIGURE 5 | Time courses of CO_2 and ethanol production with respect to LB **(A)**, LBEF **(B)** and LBLP **(C)** under anaerobic conditions. LB, *Lentilactobacillus buchneri*; LBEF, LB mixed with *Enterococcus faecium*; LBLP, LB mixed with *Lactiplantibacillus plantarum*.

optimal solution of the Bolza equation (i.e., the term of min.$(t_f - t_0)_{pH\downarrow}$ in Eq. 3).

Formations of CO_2 and EtOH

Three time courses of CO_2 and EtOH formation (**Figure 5**) were recorded from the LB (A), the LBEF (B), and the LBLP (C). The fermentation governed by the LBLP yielded the lowest CO_2 (48.12 mg) and EtOH (14.55 mg) in the period of 48 h. In contrast, the fermentation with the LB or the LBEF yielded more than twice as much CO_2 (86.21–90.72 mg) and EtOH (28.14–32.10 mg). **Figure 6** shows the normalized CO_2 production ($\overline{CO_2}$, **Figure 6A**) and $\overline{EtOH}$ (**Figure 6B**) from the three samples. For the LBLP, the $\overline{CO_2}$ reached 0.8 of the final production in 24.5 h, while the $\overline{CO_2} =$ 0.8 for the LB was 28 h or for the LBEF was 31 h. Similarly, the transition times of $\overline{EtOH}$ increased to 0.8 for the LBLP, LB, and LBEF at 12.3, 19.5, and 21.8 h, respectively. In contrast, the rate of EtOH increase was greater than that of CO_2 in all the samples.

Metabolic Sensitivity to pH

Figure 7 shows stepwise tracing ($t_i - t_{i-1} = 2$ h, Eq. 6) for the temporal production of both CO_2 and EtOH. In the initial period of fermentation (pH > 5), the increasing ΔCO_2 and ΔEtOH reflected the increasing microbial activity for all microbes. A turning point of ΔCO_2 and ΔEtOH occurred around pH 5. Below pH 5, the metabolic activity decreased as the pH declined. After the pH declined to 4, both ΔCO_2 and ΔEtOH reached minima and then achieved steady state. The patterns in **Figure 7** not only characterize the metabolic sensitivity of these microbes to pH but also contain the dynamic information of the acidification process from each sample. For the LBLP sample, pH_{LBLP} decreased to 5 in less time (4.5 h), resulting in only three data points of ΔCO_2 (t_i - t_{i-1} = 2 h) while pH $\geq$ 5. In contrast, seven data points of ΔCO_2 for LB and LBEF were recorded due to the longer periods of pH $\geq$ 5 (pH_{LB}, 11.5 h; pH_{LBEF}, 10 h). The patterns of EtOH (**Figures 7B,D**) had similar temporal implications. Because the turning points of ΔCO_2 and ΔEtOH at pH 5 correlated for all the microbial samples (**Figure 7**), the general effect of pH on the metabolic activity can be estimated. **Figure 8** presents four piecewise linear regressions corresponding to ΔCO_2 (**Figure 8A** pH < 5, **Figure 8B** pH > 5) and ΔEtOH (**Figure 8C** pH < 5, **Figure 8D** pH > 5), related to the three microbial samples.

Role Partition of Each Strain

In functional space, the time courses of both pH_{LBLP} and pH_{LBEF} (**Figure 3**) are mathematically decomposable in relation to the time course of pH_{LB}. With the use of Eq. 7 together with the time course of pH_{LB} (**Figure 3**) as reference, **Figure 9** shows these separate time courses for pH_{LP} and pH_{EF} over the initial 12 h of the fermentation. We presented the decomposed data in the early stage (0–12 h) because pH_{LBLP} reached a plateau within 12 h $\leq t \leq$ 48 h (**Figure 3**). The regression demonstrated that the LP species played an exponentially accelerating role (R^2 = 0.982, RMSE = 0.053) in the acidification process over pH ranging from 6.23 to 5.0. The major contribution of LP to the fermentation process was observed in the initial period of 0–6 h, i.e., pH $\geq$ 5.5. Subsequently, the accelerating role of the LP attenuated quickly as the pH decreased, and this could be attributed to the stronger suppression from the increasing organic acid in the fermentation medium. Alternatively, the separate role of the EF is a straight line, perpendicular to the pH axis at the initial point (pH_0 = 6.23). This demonstrates that the role of the EF in accelerating the acidification process is negligible, not an optimal solution for Eq. 3.

DISCUSSION

Both the shortest acidification process (min. t_f in section "Acidification Characteristics") and the minimum gas production (see section "Formations of CO_2 and EtOH") resulted from the LBLP, and therefore, this is a time-energy optimal fermentation reaction (Sargent, 2000). Moreover, the time course of pH_{LBLP} (**Figure 3**) is the optimal trajectory of the fermentation process, and the exponential equation of the LP (**Figure 9**) is the optimal control function ($\hat{u}_{m-best}$) determined. The paired optimal solutions were found experimentally and subject to the Bolza equation (Eq. 3) (Clarke, 1976).

As expected, lactic acid was the primary metabolite of LAB fermentation (**Table 3**). Our experimental data

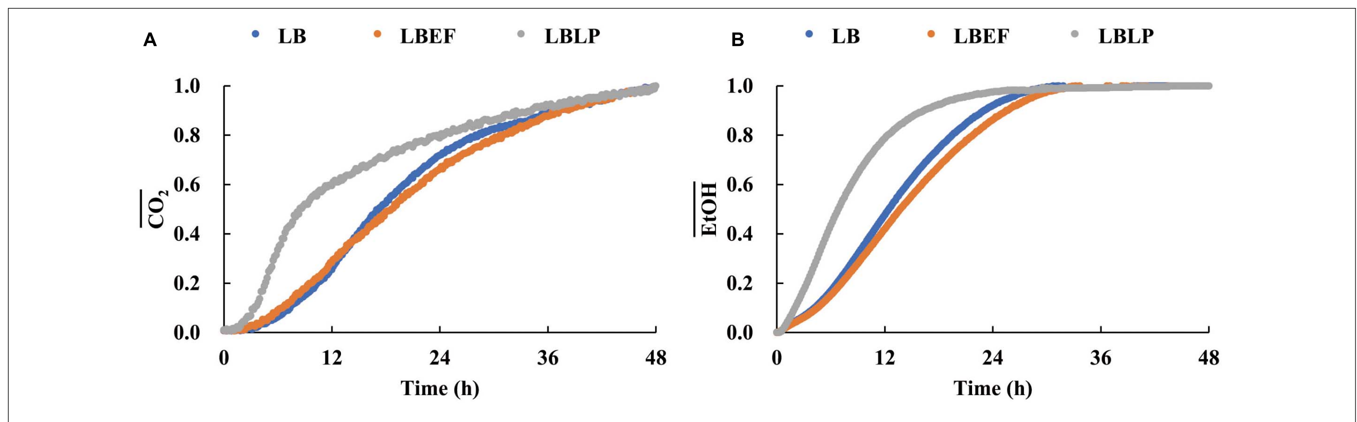


FIGURE 6 | Relative variations of CO_2 **(A)** and EtOH **(B)** productions of the three samples.

FIGURE 7 | The relationships between the pH of the three microbial samples and the differential variables of both CO_2 production **(A,C,E)** and EtOH production **(B,D,F)**, where $\Delta CO_2 = CO_2\ (t_i) - CO_2\ (t_{i-1})$ and $\Delta EtOH = EtOH(t_i) - EtOH(t_{i-1})$, both calculated with $t_i - t_{i-1} = 2$ h, $i = 0, 1, \ldots, n$. The arrows denote the time course of decreasing pH.

supported the hypothesis that pH was primarily dominated by variations in lactic acid during the LAB fermentation process (Kung et al., 2018a) from two notes: (i) the *Lp. plantarum* strains DSM 3676 and DSM 3677 (LP), the facultative heterofermentative strains, played a major role in producing lactic acid during the initial 0–6 h of the fermentation (**Figures 3, 9**). At $t = 6$ h, the $pH_{LBLP} = 4.62$ indicated that most lactic acid had already been formed. (ii) The pKa of acetic acid (4.75) is higher than that of lactic acid (3.86), reflecting it being a 10 times weaker acid than lactic acid (Danner et al., 2003; Graves et al., 2006; Kung et al., 2018a).

Our data of the relationship between CO_2 and pH (**Figures 7, 8**) support a value of pH 5 as a critical value governing microbial growth, with rapid (pH > 5) or slower to no growth (pH < 5) during the ensiling process (Kung et al., 2003; Pahlow et al., 2003). We show this for the first time

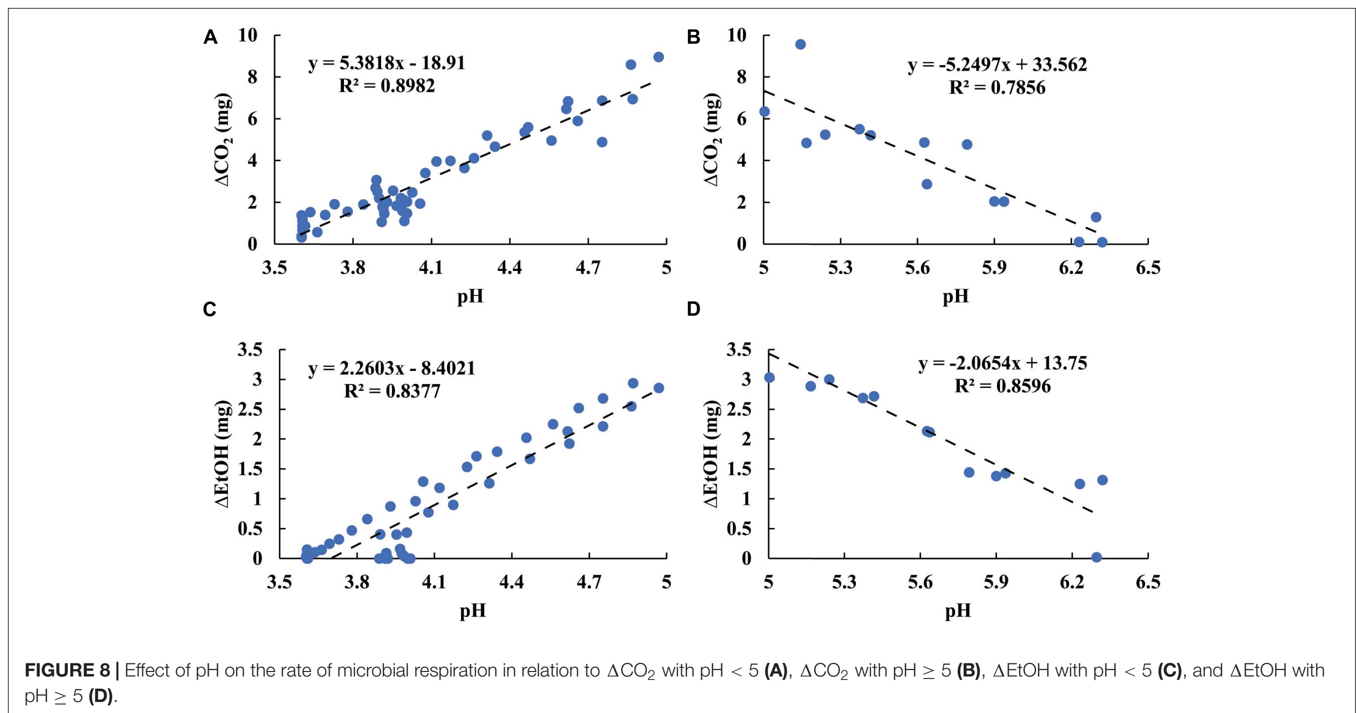


FIGURE 8 | Effect of pH on the rate of microbial respiration in relation to ΔCO_2 with pH < 5 **(A)**, ΔCO_2 with pH ≥ 5 **(B)**, ΔEtOH with pH < 5 **(C)**, and ΔEtOH with pH ≥ 5 **(D)**.

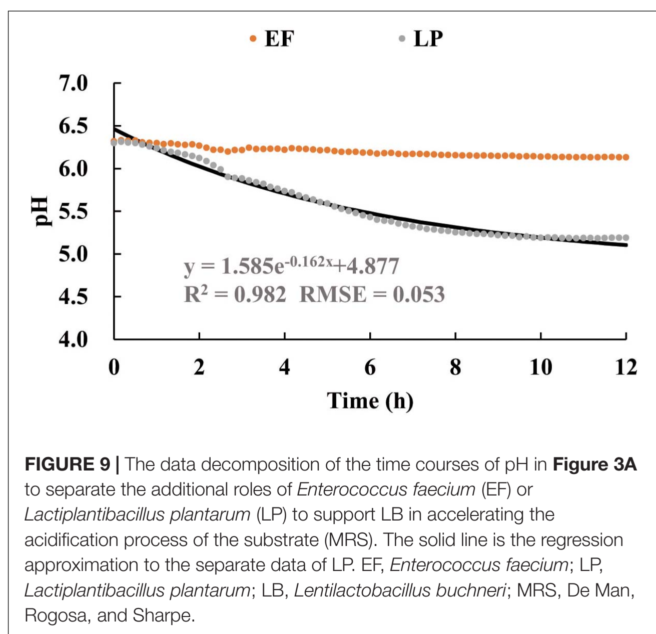


FIGURE 9 | The data decomposition of the time courses of pH in **Figure 3A** to separate the additional roles of *Enterococcus faecium* (EF) or *Lactiplantibacillus plantarum* (LP) to support LB in accelerating the acidification process of the substrate (MRS). The solid line is the regression approximation to the separate data of LP. EF, *Enterococcus faecium*; LP, *Lactiplantibacillus plantarum*; LB, *Lentilactobacillus buchneri*; MRS, De Man, Rogosa, and Sharpe.

using the dynamics of microbial respiration over the course of fermentation. This had previously been suggested from an *ex situ* determination of microbial counts (Oliveira et al., 2017). Our *in situ* method obviates the process of plate-culture counts, which create assessment delays of several days (Wilkinson and Muck, 2019) and may require multiple samplings over time. In contrast to the *ex situ* method, our MSMB provides both real-time anaerobic measurements of CO_2 and EtOH productions and instantaneous microbial activity.

A few studies tested silage inoculants using MRS broth or the aqueous extract of silage crop (Oude Elferink et al., 2001; Danner et al., 2003; Holzer et al., 2003; Graves et al., 2006; Arasu et al., 2015; Blajman et al., 2020). Fermentation characteristics, such as production of organic acids, decreasing dynamics of pH, and CO_2 production, depend on medium composition (Danner et al., 2003; Blajman et al., 2020). The next step of our study is to replace MRS broth with the aqueous extract of silage, creating a testing condition that may be closer to the natural culture of silage ensiling for LAB. However, the effect of the buffering capacity of silage crop on pH is inherent (Kung et al., 2003; Pahlow et al., 2003; Shan et al., 2021) and should be taken into account. Unfortunately, this effect cannot be evaluated when either MRS broth or the liquid extraction of silage crop is used as fermentation medium.

High concentrations of EtOH are usually attributed to large numbers of yeasts (Kung and Ranjit, 2001; Kung et al., 2018a). However, the silage containing LB (Oude Elferink et al., 2001) produced EtOH from anaerobic degradation of lactic acid in corn silage. Kung and Ranjit (2001) noted the high EtOH from treated barley silage, but not from the control. The EtOH data of our study from the MRS broth support the previous observations. Moreover, in this study, EtOH was measured *in situ* as the gaseous concentration in the sealed jar, which by Henry's law is directly proportional to the concentration of EtOH dissolved in the fermentation medium (Sun et al., 2015). While the partition coefficient of Henry's law is temperature-dependent, the fermentation here was carried out at constant temperature (30°C).

During the ensiling process, the CO_2 recorded in the fermentation phase consists of two parts, one from the initial aerobic phase and one from anaerobic fermentation

(Li et al., 2017). It is challenging to separate them into two CO_2 pools for the different phases (Shan et al., 2019). In this study, the measured CO_2 (**Figures 5–8**) derived completely from anaerobic heterofermentative LAB fermentation because the MSMB provided a manageable environment between anaerobic and aerobic seals. This is also an advantage of the MSMB over the ensiling experiment with the natural culture of silage.

It is not surprising that **Figures 3A,B** had similar patterns. Members of the family Lactobacillaceae are anaerobic, but the majority of species are oxygen tolerant to some degree and often completely. Only very few species of LAB were observed to react to O_2 (Condon, 1987). On the technical side, **Figure 3** demonstrates that the multi-sensor instrument presented here is suitable for both anaerobic and aerobic experiments. Since the aerobic stability of silage refers to a de-acidification process governed by fungus in silage (Wilkinson and Davies, 2012; Kung et al., 2018a), and the aerobic deterioration of silage is commonly associated with changes in temperature, pH, CO_2 production, and O_2 consumption (Honig, 1990; Muck and Pitt, 1994; Weinberg and Ashbell, 2003; Sun et al., 2015; Robinson and Swanepoel, 2016), this novel MSMB could also be useful to qualitatively observe the role of acetic acid in inhibiting fungal growth during aerobic phases of silage production.

CONCLUSION

We have devised an MSMB to facilitate preselection of microbes as silage inoculants. We demonstrate successful screening of the dynamics of the acidification process, gas production, and metabolic activity from the MRS-based LAB fermentation, using *in situ* simultaneous measurements of pH, CO_2, and EtOH. We have further used this novel information to introduce an optimal control model, using signal decomposition, for selecting candidate microbes. Future studies are planned to evaluate this novel prototype with aqueous extracts of common silage materials during anaerobic LAB fermentation and to extend its applicability to silage aerobic stability.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AM and YS designed the study. WBu, GS, WBe, CM, and YS devised the instrument. GS, VR, YW, and YS performed the experiment. YS designed the mathematical frame. GS and YS designed the data process. GS made the statistical analysis. VR conducted the chemical analysis. GS, AM, AL, DG, and YS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Microbial Community and Fermentation Characteristics of Native Grass Prepared Without or With Isolated Lactic Acid Bacteria on the Mongolian Plateau

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This study aimed to isolate and identify lactic acid bacteria (LAB) from the native grass and naturally fermented silage from the Mongolian Plateau. The effect of selected strains on bacterial community and quality of native grass silage was also studied. Strains XM2, 265, and 842 could grow normally at 15°C–30°C, pH 4.0–8.0, and NaCl 3 and 6.5%; they were identified as *Lactiplantibacillus plantarum* subsp. *plantarum*, *Pediococcus acidilactici,* and *Latilactobacillus graminis*, by sequencing 16S rRNA, respectively. The three strains (XM2, 265, and 842) and one commercial additive (L) were used as inoculants and singularly added to the native grass. Compared to the control, the dry matter content was significantly ($p < 0.05$) lower in L and XM2 groups. The water-soluble carbohydrate content was significantly ($p < 0.05$) higher in control than in other groups. Compared with the control, the crude protein and ammonia nitrogen contents were significantly ($p < 0.05$) higher and lower in the LAB-treated groups, and the acid and detergent fiber contents were significantly ($p < 0.05$) reduced in the L and XM2 groups than those in other groups. There was a significant ($p < 0.05$) difference in the pH value, lactic acid content, and lactic acid-to-acetic acid ratio in L and XM2 groups than in other groups. Compared with the control, the number of LAB was significantly ($p < 0.05$) higher in LAB-treated silages, whereas no significant ($p > 0.05$) differences were observed in yeast and aerobic bacteria in all groups. Compared to the control, the Shannon index was significantly ($p < 0.05$) reduced. Simpson and Chao1 were significantly ($p < 0.05$) increased. Principal coordinate analysis based on the unweighted UniFrac distance showed clear separation of the bacterial community in fresh materials and LAB-treated silages. Besides, compared to the control, the principal coordinate analysis of LAB-treated silages was also separate. After 30 days of fermentation, the

relative abundance of *Firmicutes* increased and was the primary phylum in all silages. Compared with the control, the abundance of *Firmicutes* and *Proteobacteria* was significantly ($p < 0.05$) higher and lower in L and XM2 groups. In contrast, no significant differences were observed among control, 265, and 842 groups. At the genus level, the relative abundance of *Lactobacillus*, *Enterobacter*, *Pediococcus,* and *Weissella* was increased and dominated the native grass fermentation. Compared with the control, the abundance of *Lactobacillus* was significantly ($p < 0.05$) higher in L, XM2, and 842 groups, while no significant ($p > 0.05$) differences were observed between the control and 265 groups. The abundance of *Pediococcus* was higher than that in other groups. Consequently, the results demonstrated that LAB significantly influenced silage fermentation by reconstructing microbiota, and *Lactobacillus* was the dominant genus in the native grass silages. Furthermore, the results showed that strain XM2 could effectively improve the silage quality, and it is considered a potential starter for the native grass silage.

Keywords: isolation, lactic acid bacteria, bacterial community, native grass, fermentation quality

INTRODUCTION

Native grasslands are widely distributed in the north and west of China, including the Mongolian Plateau, Qinghai, and the Tibet Plateau, are an essential resource in animal production, and grow well in fall and autumn, providing sufficient nutrition and biomass for animals (Du et al., 2019). Hay is a traditional method for maintaining forage, while the disadvantages of native grass hay, including hay quality and palatability, make it hard to shake off the seasonal and yearly imbalance of available forage (Yan et al., 2019). Ensiling is a traditional way for preservation of animal feed and green forage crops because it can supply forages for animals year-round, effectively reduce the nutrition loss of forages, and prolong storage time (Pahlow et al., 2003). However, the moisture and water-soluble carbohydrate (WSC) content and the number of lactic acid bacteria (LAB) were lower than the requirement for a well-preserved silage (You et al., 2021). Therefore, it is difficult to produce high-quality silage of native grass using natural fermentation.

Generally, LAB additives are a practical method for improving fermentation quality, which is widely used for silage preparation. The LAB group has been selected from various forages, including alfalfa (Ogunade et al., 2018), *Elymus nutans* (Xu et al., 2018), oat (Romero et al., 2017), King grass (Shah et al., 2018), *Moringa oleifera* (Wang Y. et al., 2018), native grass (You et al., 2021), and Teff (Tilahun et al., 2018). Ensiling involves complex microbial interactions, and the microbial ecology associated with silages was conducted using classical microbial techniques (Keshri et al., 2019). Studies on the microbial composition during silage fermentation have shown that *Lactobacillus* often plays an important role during the later stages of ensiling and manufactures the amount of lactic acid to improve silage fermentation (Ding et al., 2019). Previous studies also found that *Lactobacillus*, *Weissella*, *Pseudomonas*, and *Leuconostoc* spp. in silage samples were the dominant genera (Guan et al., 2018; Keshri et al., 2019; Yang et al., 2019; Bai et al., 2020). Although the isolation, selection, and application of LAB are critical for silage fermentation and bacterial communities, the LAB was isolated from various forages and grasses with different effects on silage fermentation by various environments (Yang et al., 2010; Zhang et al., 2015; Yan et al., 2019), but few LAB have been isolated and applied on native grass in the Mongolian Plateau. Some LAB strains were isolated from the native grass and naturally fermented silage in the Mongolian Plateau in our research. These strains grew under low-pH conditions in an anaerobic environment, producing more lactic acid, and widely used carbohydrates. Still, the effects of these strains on silage fermentation of native grass remain unclear.

Consequently, this study aimed to select and identify LAB strains using physiological and morphological tests and molecular methods from native grass, and its effects on the bacterial community and the silage quality of native grass were also studied.

MATERIALS AND METHODS

Lactic Acid Bacteria Strains

A total of 23 LAB strains were isolated from 35 native grass and naturally fermented silage samples. The grass was harvested at the milk stage from typical and meadow steppe in Inner Mongolia Plateau, China, in August 2018 and 2019. The grassland contained typical and meadow steppe flora of HulunBuir, Inner Mongolia, with the Giant Feathergrass (*Stipa gigantea* Link.) and Chinese Leymus (*Leymus chinensis* [Trin.] Tzvel.), and the Baical Needlegrass (*Stipa baicalensis* Roshev.) and Chinese Leymus (*Leymus chinensis* [Trin.] Tzvel.) being the dominant species, respectively. The 10-g silage samples from each silage sample were homogenized with 90 ml of distilled water; serial dilutions were used for potential LAB culturing and purification by streaking on de Man, Rogosa, Sharpe (MRS) agar (Difco Laboratories, Detroit, MI, United States) four times at 30°C for 48 h and stored at −80°C in MRS broth with 20% glycerol.

Physiological and Morphological Tests

Gram staining, catalase activity, and gas production from glucose were determined as previously reported (Duan et al., 2008; You et al., 2021). Growth at different temperatures and pH environments (HCl or NaOH were used for adjusting pH) was based on the method of Cai et al. (1999c). Salt tolerance was determined using the method of Wang S. et al. (2018). The growth was measured and evaluated using optical density (OD) value (You et al., 2021). API 50 CH (BioMérieux, Marcy l' Etoile, Lyons, France) was used to determine the carbohydrate assimilation (Cai et al., 1999a).

Lactic Acid Bacteria Identification by 16S rRNA Sequencing

To extract the DNA of the screened strains, Bacterial DNA kit was used (Tiangen Biotech Co., Ltd., Beijing, China). The primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACT-3′) were used in the polymerase chain reaction (PCR) (Cai et al., 1999b). The species identification was conducted as previously reported (Zhang et al., 2015). The 16S rRNA sequences were identified using BLAST analysis on GenBank (Ennahar et al., 2003).

Preparation of Silage

Native grass was harvested at the milk stage on August 20, 2020, in Chifeng, China. The native grassland was meadow steppe flora of Baarin Left Banner, Inner Mongolian Plateau, including Giant Feather Grass (*S. gigantea* Link.), Chinese Leymus [*L. chinensis* (Trin.) Tzvel.], and Dahurian Bushclover [*Lespedeza davurica* (Laxm.) Schindl] as the dominant species. Strains XM2, 265, and 842, and commercial LAB additive (defined as L, CH, *Lactobacillus plantarum*, Snow Brand Seed Co., Ltd, Sapporo, Japan) were used as inoculants. All LAB strains were added singularly at 10^5 cfu/g fresh materials (FM), and the control was added to the same volume of distilled water (Wang Y. et al., 2018; You et al., 2021). The FM was chopped using a manual forage chopper (Xianglong Co., Ltd., Linyi, China) with 2- to 3-cm lengths and was immediately moved to the laboratory. About 500 g of FM in a plastic polyethylene bottle (1 L capacity) and air were eliminated. Each treatment was prepared with three replicates and stored at ambient temperature for 30 days.

Chemical and Fermentation Characteristics Analysis

Dry matter content of raw material and 30 days of fermentation of native grass silage samples was determined by drying samples in a forced-air oven at 65°C for 72 h. However, based on the Association of Official Analytical Chemists, crude protein (CP) content was determined (Association of Official Analytical Chemistry, 1990). Fiber fractions were determined according to the method of Van Soest et al. (1991). The WSC content was determined as previously reported (Murphy, 1958).

The 10-g sample was added to 90 ml of distilled water and remained for 24 h at 4°C in a refrigerator, and the extract was filtered through four layers of cheesecloth. The pH value and organic acid contents were determined using the method of You et al. (2021). The ammonia nitrogen (NH_3-N) content was assessed as described by Kleinschmit et al. (2005).

Microbial Composition Analysis

The microbial compositions of FM and silages were analyzed using the plate count method. Under anaerobic conditions on MRS agar incubated at 30°C, the counts of LAB were quantified for 48 h (You et al., 2021); coliform bacteria, aerobic bacteria, mold, and yeast were calculated using the previously reported method (You et al., 2021) and expressed on colony-forming units (cfu)/g of FM.

DNA Extraction, Polymerase Chain Reaction Amplification, and Sequencing

Bacterial community genomic DNA was extracted from native grass and silage samples using the E.Z.N.A.® sample DNA kit (Omega Bio-tek, Norcross, GA, United States). The NanoDrop 2000 UV-vis Spectrophotometer (Thermo Scientific, Wilmington, United States) was used in determining the concentration and purity of the extracted DNA and 1% agarose gel electrophoresis was used to analyze the quality of extracted DNA (Bai et al., 2020). All extracted DNA samples were frozen at −20°C for analysis.

Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) performed PCR amplification and bioinformatic analysis. The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified using primer 338F (5′-ACTCCTACGGGAGG CAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 16S amplification was conducted according to the description of Tian et al. (2017). Raw sequence data were uploaded to the NCBI's Sequence Read Archive under study accession number PRJNA758799.

Statistical Analyses

Paired-end reads were assigned to samples based on their unique barcode, truncated by cutting off the barcode and primer sequence and merged using FLASH (v1.2.8) (Mago and Salzberg, 2011). Under specific filtering conditions, quality filtering on raw tags was performed to obtain high-quality clean tags according to fqtrim (v0.94) (Li et al., 2021). The chimeric sequences were filtered using Vsearch software (v2.3.4) (Rognes et al., 2016), and the high-quality sequences with $\geq$ 97% similarity were assigned to the same operational taxonomic units (OTUs) (Li et al., 2021). Taxonomic summaries were conducted by calculating the relative abundance across samples and normalizing to 100%. Alpha diversity and beta diversity were computed by normalizing to the same sequences randomly using QIIME2. OmicStudio tools[1] were used to perform most of the graphics drawing. The chemical composition, silage quality, and microorganism population of silages were performed using a one-way analysis of variance with three replicates. Duncan's tests separated significant differences and were considered statistically significant at the 5% probability level (SAS Inc, 2007).

[1] https://www.omicstudio.cn/tool

RESULTS

The Selection of Isolated Lactic Acid Bacteria Based on Morphological and Physiological Tests

The selection of isolated LAB based on morphological and physiological tests is indicated in **Table 1**. All isolated strains were Gram-positive and catalase-negative, the XM2 strain was rod-shaped, and strains 265 and 842 were cocci-shaped. All strains normally grew at 15 and 30°C, pH 4.0–8.0, while strain 265 normally grew at 50°C, and strains XM2 and 842 grew normally at 10°C. Under NaCl concentrations of 3.0 and 6.5%, all isolated strains grew normally. According to the results of gas for glucose, the three strains possessed similar fermentation patterns and were homofermentative, while different ferment carbohydrates are indicated in **Table 2**. The three isolated strains that could ferment carbohydrates in API are listed in **Table 2**. In this study, the GenBank data library was used to analyze the similarity through BLAST[2], strain XM2 possessed a higher similarity with *L. plantarum*, strain 265 possessed a higher similarity with *Pediococcus acidilactici*, and strain 842 possessed a higher similarity with *Lactobacillus graminis*, with 99.87, 99.58, and 99.63% similarities in their 16S rRNA gene sequence, respectively (**Table 3** and **Supplementary Figure 1**). The strains' nucleotide sequences were also transferred to GenBank with accession numbers MT358326, MT358327, and MT358333 for XM2, 265, and 842, respectively. Based on the new classification of *Lactobacillus* genus, strains XM2, 265, and 842 were identified as *Lactiplantibacillus plantarum* subsp. *plantarum*, *P. acidilactici*, and *Latilactobacillus graminis*, respectively.

[2]blast.ncbi.nlm.nih.gov

The Chemical Compositions and Microbial Population of the Fresh Materials Before Ensiling

The chemical compositions and microbial population of the substrates prior to ensiling are shown in **Table 4**. The moisture content of native grass was 40.66%, and WSC, CP, NDF, and ADF were 4.36, 11.74, 58.15, and 30.07% on a DM basis, respectively. Microbial populations in the native grass for LAB, coliform bacteria, aerobic bacteria, and yeasts were 4.01, 6.93, 7.68, and 7.41 $\log_{10}$cfu/g FM, respectively. Mold was not detected in native grass.

TABLE 1 | The selection of isolated lactic acid bacteria on the base of the morphological and physiological tests.

Items	XM2	265	842
Shape	Rod	Cocci	Cocci
Gram stain	+	+	+
Gas for glucose	−	−	−
Catalase	−	−	−
Fermentation type	Homo	Homo	Homo
Growth at temperature (°C)			
5	w	−	w
10	+	−	+
15	+	+	+
30	+	+	+
45	w	+	+
50	w	+	w
Growth at pH			
3.0	w	−	−
3.5	+	+	−
4.0	+	+	+
5.0	+	+	+
6.0	+	+	+
7.0	+	+	+
8.0	+	+	+
Growth in NaCl (%)			
3.0	+	+	+
6.5	+	+	+

+, positive;−, negative; w, weakly positive. Homo; homofermentative.

TABLE 2 | The characteristics of isolated lactic acid bacteria on the base of carbohydrate fermentation.

Item	XM2	265	842
L-Arabinose	−	−	+
Ribose	+	+	+
D-Xylose	−	+	−
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
D-Mannitol	+	−	−
D-Sorbitol	+	−	−
Methyl-αD-Mannopyranoside	+	−	−
N-Acetyl Glucosamine	w	w	+
Amygdalin	+	w	w
Arbutin	w	w	−
Esculin	+	+	+
Salicin	w	w	w
Cellobiose	+	+	+
Maltose	+	−	−
Lactose	+	−	−
Melibiose	+	−	−
Sucrose	+	−	−
Trehalose	+	+	+
Melezitose	+	−	−
Raffinose	+	−	−
Gentiobiose	w	w	w
D-Tagatose	−	w	−
D-Arabitol	w	−	−
Gluconate	w	−	−

All strains gave negative results for glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, methyl-αD-glucopyranoside, inulin, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, L-arabitol, 2-keto-gluconate, and 5-keto-gluconate. +, positive; -, negative; w, weakly positive.

Chemical Compositions, Silage Quality, and Microbial Populations of Ensiling

The chemical compositions, silage quality, and microbial populations on 30 days of native grass ensiling are indicated in **Table 5**. There were significant ($p < 0.05$) differences in DM contents in the L and XM2 groups than in the control, while no significant ($p > 0.05$) differences were observed among control, 265, and 842 groups. The WSC content was significantly ($p < 0.05$) greater in control than in other groups. Compared to control, the NH_3-N and CP contents were significantly ($p < 0.05$) greater and lower than in the L and XM2 groups, respectively. The NDF and ADF contents also significantly decreased in L and XM2 groups compared to that in the control. After 30 days of fermentation, compared to control, the pH in inoculated silages was significantly ($p < 0.05$) lower, and lactic acid (LA) content and lactic acid-to-acetic acid ratio were significantly ($p < 0.05$) increased. The number of LAB was significantly ($p < 0.05$) greater in inoculated silages than in control. No significant ($p > 0.05$) differences were observed in yeast and aerobic bacteria in all groups, and coliform bacteria and mold were lower than detectable in all groups.

Microbial Diversity of Fresh Materials and Native Grass After Ensiling

This study also analyzed the bacterial microbiota in silages and identified the species-discriminatory taxa. The sequencing information and bacterial diversity analysis are indicated in **Supplementary Table 1**. Compared to FM, the OTUs, Shannon, and Chao1 decreased significantly ($p < 0.05$). The Good's coverage of all groups was more than 99%. Compared to the control, the Shannon index was significantly ($p < 0.05$) reduced, and Simpson and Chao1 were significantly ($p < 0.05$) increased.

Next, principal coordinate analysis (PCoA) was conducted based on the unweighted UniFrac distance to determine whether the microbial community structure changed in FM and silages (**Figure 1**). The PCoA plot exhibited a clear separation of bacterial community in FM and silages; besides, L, XM2, and other LAB-treated groups were also separate.

The relative abundance of bacteria in FM and silages of 30 days of ensiling are indicated in **Figure 2**. In FM, the dominant phyla of native grass were *Proteobacteria* and *Actinobacteriota* (**Figure 2A**). After 30 days of ensiling, the relative abundance of *Firmicutes* increased and was the primary phylum in all silages (80%, **Figure 2B**), and significant ($p < 0.05$) differences were observed among FM and silages in *Firmicutes*, *Proteobacteria*, and *Actinobacteriota* at the phylum level (**Figure 2B**). Compared to control, the abundance of *Firmicutes* and *Proteobacteria* was significantly ($p < 0.05$) higher and lower in L and XM2 groups, whereas no significant differences were observed among control, 265, and 842 groups. In FM, the dominant phyla of native grass were *Pantoea*, whose abundance was more than 50% (**Figure 2C**). After 30 days of fermentation, the relative abundance of *Lactobacillus*, *Enterobacter*, *Pediococcus,* and *Weissella* was increased and dominated the native grass fermentation among silages (80%, **Figure 2B**), and significant ($p < 0.05$) differences were observed among FM and silages in *Lactobacillus*, *Enterobacter*, *Pediococcus*, and *Weissella* (**Figure 2D**). Compared to control, the abundance of *Lactobacillus* was significantly ($p < 0.05$) higher in L, XM2, and 842 groups, while no significant ($p > 0.05$) difference was observed between control and 265 groups. The abundance of *Pediococcus* was the ($p < 0.05$) highest than in other groups.

The linear discriminant analysis effect size (LefSe) was applied to explore the relative richness ($p < 0.05$, LDA > 3.0) of silages (**Figure 3**). The *Lactobacillus* was enriched in the XM2 group, and *Pediococcus* was enriched in the 265 group.

TABLE 3 | The results of isolated lactic acid bacteria on the base of 16S rRNA gene sequences.

Strain	Accession number	16S rRNA gene sequencing data (closest relative)	Similarity (%)
XM2	NR_115605.1	*Lactobacillus plantarum* JCM 1149	99.87
265	NR_042057.1	*Pediococcus acidilactici* DSM 20284	99.58
842	NR_042438.1	*Lactobacillus graminis* G90	99.63

Chemical composition and microbial population of native grass prior to ensiling.

TABLE 4 | Chemical composition and microbial population of native grass prior to ensiling.

Items	Native grass
Dry matter (%)	59.34
Water-soluble carbohydrates (% DM)	4.36
Crude protein (% DM)	11.74
Acid detergent fiber (% DM)	30.07
Neutral detergent fiber (% DM)	58.15
Lactic acid bacteria (log_{10} cfu/g FM)	4.01
Yeast (log_{10} cfu/g FM)	7.41
Aerobic bacteria (log_{10} cfu/g FM)	7.68
Coliform bacteria (log_{10} cfu/g FM)	6.93
Mold (log_{10} cfu/g FM)	ND

DM, dry matter; FM, fresh matter; cfu, colony-forming units; ND, not detected.

DISCUSSION

Forage crops, silages, and dairy products have been found in various LAB species, and many isolates have been identified as the *Lactobacillus* group (You et al., 2021). Previously published studies also showed that *Lactobacillus* is the primary microorganism on forages and silages (Cai et al., 1999c). However, it is challenging to identify the species among differentiated species by morphological, physiological, and biochemical tests (Chen et al., 2013). The 16S rDNA sequence analysis identifies organisms by genus and species (Ennahar et al., 2003).

Similarly, strains XM2, 265, and 842 at pH 4.0 could grow normally and strain XM2 weakly grew at pH 3.0. These results showed that strain XM2 had a high tolerance to acidity and the ability to grow and thrive in low-pH environments, which agree

with the findings that *L. plantarum* displayed high resistance to a low-pH environment (Wang S. et al., 2018). Additionally, these isolated strains grew normally from 5°C to 30°C. While significant differences were observed among these strains, strains XM2 and 842 grew normally at 10°C, and strain 867 grew well at 50°C, which could be attributed to the unique environment, long periods of natural selection, and evolution on the Inner Mongolian Plateau (You et al., 2021). Therefore, the unique traits of these isolated LAB strains could have applications as additives on silage fermentation. What is more, strain XM2 could ferment more substrates than the other strains, which is consistent with the previous report that shows that the *L. plantarum* group could ferment a wide variety of carbohydrates (McDonald et al., 1991).

The moisture content of the grass is one of the essential factors that can directly influence the silage fermentation quality. In this study, the moisture content of native grass agrees with the previous report that shows the range of 44.93–47.51% in meadow steppe on the Mongolian Plateau (Hou et al., 2017). The plant diversity and environment may contribute to the difference. The WSC content was lower than an adequate WSC concentration that good silage needs WSC content higher than 5% on DM for LAB fermentation (Amer et al., 2012). This result follows the previous study on the low WSC content in native grass (Hou et al., 2017). The CP, ADF, and NDF contents followed those of Du et al. (2019), who discovered the native grass with a lower CP content and higher NDF and ADF contents.

Generally, the silage fermentation process and fermentation quality were determined by the counts and species of epiphytic LAB (Yan et al., 2019). A previously published study showed that the well-preserved silages need the numbers of *Lactobacilli* to be at least 10^5 cfu/g FM of ensiling (Cai et al., 1999c). However, in this study, the numbers of LAB and undesirable microorganisms were lower (10^5 cfu/g) and higher (10^4–10^8 cfu/g) in FM than the required values, which could lead to poor fermentation quality. Additionally, LAB groups also included other species that may have few effects on fermentation. Consequently, the use of LAB additives is necessary for native grass silage; not only the low moisture, WSC content, and LAB counts, but also the growth of undesirable microorganisms was inhibited during the early stages of fermentation.

The DM content was lower in the inoculated groups than in the control in this study. Besides, the WSC content was significantly reduced in inoculated silages compared to the control silage, which agrees with Kleinschmit and Kung (2006), who found that grass silages with inoculant had a lower WSC content than that in the control. The WSC contents were fermented by LAB and transformed into organic acids, ethanol, and carbon dioxide by microorganisms during silage fermentation (Muck et al., 2017; Wang S. et al., 2018). The acid hydrolysis during ensiling also reduced the DM content (Zhao et al., 2018). Therefore, the DM, WSC, NDF, ADF, and pH decreased in inoculated silages. The CP and NH_3-N contents in control were lower and greater than those in the inoculated silages, reflecting the growth of undesirable microorganisms and the accumulation of NH_3-N during ensiling (Dong et al., 2020). The higher CP and lower NH_3-N contents in inoculated silages may be attributed to the lower pH, which inhibits the growth and activities of undesirable microorganisms (Arriola et al., 2011; Heinritz et al., 2012).

The pH value in inoculated silages was lower than that in the control, while the lactic acid in the control was lower than that in L, XM2, and 265 groups. These results may be attributed to a high amount of LA with reducing pH values in the anaerobic environment (Fiyla, 2003). A previous study indicated that a pH of less than 4.20 could inhibit the growth of harmful bacteria and ensure fermentation quality (Wang et al., 2019). The pH value of the control was significantly higher than that of inoculated silages, and the pH value was 4.42. The lower number of LAB and

TABLE 5 | Chemical compositions, fermentation characteristics, and microbial populations on 30 days of ensiling.

Item	CON	L	XM2	265	842	SEM	*p*-value
Dry matter (%)	58.69a	56.84b	56.61b	57.77ab	57.80ab	0.247	0.0195
WSC (g/kg)	3.34a	2.29cd	2.07d	2.36c	2.71b	0.123	<0.0001
Crude protein (% DM)	9.58d	10.36b	10.73a	10.04c	9.85c	0.110	<0.0001
NH_3-N (g/kg)	6.28a	5.02c	4.56c	5.52b	5.52b	0.163	<0.0001
Acid detergent fiber (% DM)	33.29a	31.61bc	31.11c	32.60ab	32.26abc	0.243	0.0114
Neutral detergent fiber (% DM)	59.27a	56.97bc	56.11c	58.68a	58.10ab	0.344	0.0019
pH	4.42a	4.10cd	4.05d	4.17bc	4.21b	0.035	<0.0001
Lactic acid (g/kg)	13.10c	21.62a	21.49a	15.46b	12.88c	1.060	<0.0001
Acetic acid (g/kg)	2.86ab	2.62b	2.38b	2.62b	3.57a	0.147	0.0677
Propionic acid (g/kg)	0.22b	0.32a	0.27ab	0.27ab	0.20b	0.016	0.0913
lactic to acidic acid ratio	4.58bc	8.41a	9.17a	6.01b	3.67c	0.614	0.0003
Lactic acid bacteria (log_{10} cfu/g FM)	7.02c	7.50b	7.75a	7.42b	7.35b	0.048	<0.0001
Yeast (log_{10} cfu/g FM)	2.89	2.45	2.58	3.11	2.17	0.191	0.5716
Aerobic bacteria (log_{10} cfu/g FM)	3.88	3.89	3.69	3.91	3.84	0.035	0.2711
Coliform bacteria (log_{10} cfu/g FM)	ND	ND	ND	ND	ND		
Mold (log_{10} cfu/g FM)	ND	ND	ND	ND	ND		

DM, dry matter; FM, fresh matter; cfu, colony-forming units; ND, not detected; CON, control group; L, commercial inoculant group; XM2, strain XM2 group; 265, strain 265 group; 842, strain 842 group. SEM, the error of the means. a–c Means within a column without a common superscript letter difference, at $p < 0.05$ level.

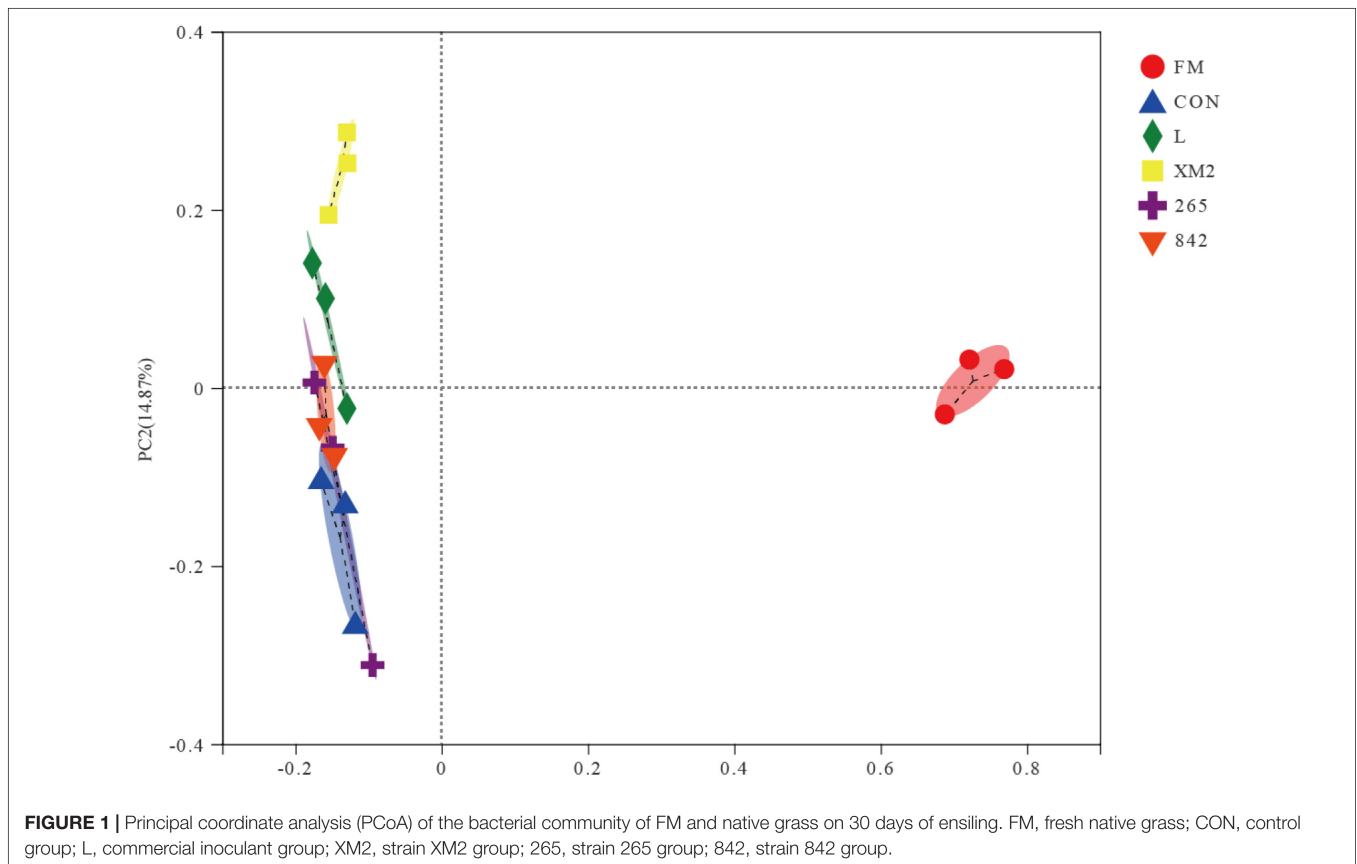

FIGURE 1 | Principal coordinate analysis (PCoA) of the bacterial community of FM and native grass on 30 days of ensiling. FM, fresh native grass; CON, control group; L, commercial inoculant group; XM2, strain XM2 group; 265, strain 265 group; 842, strain 842 group.

higher numbers of yeasts and aerobic bacteria may be the main reason because the LA and other nutrients used by yeasts, molds, and other aerobic microorganisms produce large quantities of metabolism, resulting in pH increase (Woolford, 1990). In this study, the coliform bacteria and mold were undetectable after 30 days of fermentation, which is in agreement with the report that coliform bacteria and mold were not detected after ensiling for 30 days (Dong et al., 2020). The growth of coliform bacteria and mold inhibited by lower pH was the main reason (Lima et al., 2010; Reich and Kung, 2010).

Amplicon sequencing of bacteria in fresh native grass and silage samples was performed. Good's coverage values in fresh matter and silages were higher than 99%, suggesting that sequencing could adequately reflect the dynamic change in the bacterial community (Yang et al., 2019). Compared to FM, the diversity and richness were reduced in inoculated silages, especially in inoculated silages, which follow Ogunade et al. (2018), who found that LAB additives could reduce the bacterial diversity due to the increase in the abundance of the predominant genus. The antibacterial activity that affects the composition of different bacteria and the lower pH inhibiting the growth of the desirable bacteria in this study may be the main reason (Bai et al., 2020). Compared to the control, the diversity and species richness of the microbial communities in the LAB-treated silages decreased, which agrees with previous studies that show that the addition of LAB could reduce diversity and richness (Dong et al., 2019).

The PCoA plot showed a clear separation of bacteria in FM and silages, which indicated that ensiling reconstructs the microbial community. These results followed the work of Zeng et al. (2020) that showed that the bacterial communities were distinguished in FM and silages. Besides, compared to the control, the PCoA of LAB-treated silages was also separate, which showed that the additives significantly influenced the microbial community.

This study showed that *Proteobacteria* was the most abundant phylum in FM, comprising more than 80% of the microflora, which agrees with reports that *Proteobacteria* was the dominant phylum in fresh forage (Dong et al., 2019). After 30 days of ensiling, the abundance of *Firmicutes* increased and dominated the fermentation in the native grass silages (80%), and the abundance of *Proteobacteria* decreased. These results were similar to the reports of Yang et al. (2019) and Bai et al. (2020). Compared to the control, the abundance of *Firmicutes* and *Proteobacteria* was significantly increased and lower in L and XM2 groups, whereas no significant differences were observed among control, 265, and 842 groups, which the higher microbial populations of LAB could explain.

This study showed that *Pantoea* was the major facultative aerobic genera in FM. *Pantoea* has been discovered in fresh alfalfa (Ogunade et al., 2018) and soybean (Ni et al., 2017). After 30 days of fermentation, drops in *Pantoea* abundances could be attributed to their high sensitivity to pH decline (Ogunade et al., 2018). *Lactobacillus, Pedicoccus, Weissella,* and *Leuconostoc* are

FIGURE 2 | The bacterial community of FM and native grass on 30 days of ensiling. **(A)** The bacterial community was shown at the phylum level. **(B)** The extended error bar plot displaying the significant differences among groups at the phylum level **(C)**. The bacterial community was shown at the genus level. **(D)** The extended error bar plot displaying significant differences among groups at the genus level. *Shows that the significant difference was at $p < 0.05$ level. FM, fresh native grass; CON, control group; L, commercial inoculant group; XM2, strain XM2 group; 265, strain 265 group; 842, strain 842 group.

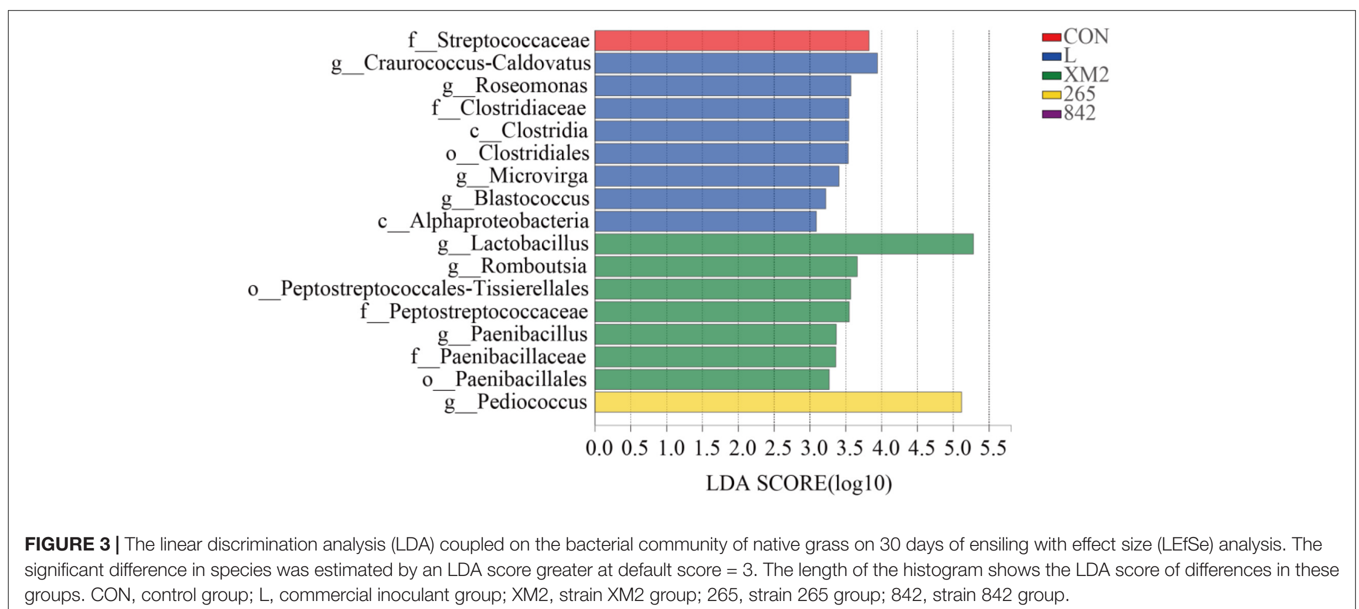

FIGURE 3 | The linear discrimination analysis (LDA) coupled on the bacterial community of native grass on 30 days of ensiling with effect size (LEfSe) analysis. The significant difference in species was estimated by an LDA score greater at default score = 3. The length of the histogram shows the LDA score of differences in these groups. CON, control group; L, commercial inoculant group; XM2, strain XM2 group; 265, strain 265 group; 842, strain 842 group.

considered as the four most predominant LAB genera responsible for driving lactic fermentation during ensiling (Pang et al., 2012; Ni et al., 2017; Guan et al., 2018; Liu et al., 2019). During ensiling, a significant shift in the bacterial community from *Proteobacteria* to *Firmicutes* could be explained by increased abundance of genera *Lactobacillus*, *Weissella,* and *Pediococcus*,

which flourished in the environmental conditions developed during ensiling (Keshri et al., 2019). Therefore, the abundance of *Lactobacillus*, *Enterobacter*, *Pediococcus,* and *Weissella* increased and dominated the silage fermentation among silages (80%). In this study, the LAB-treated silages exhibited an increase in *Lactobacillus* than in the control, which agrees with the findings that LAB-treated silages could increase the abundance of *Lactobacillus* (Liu et al., 2019; Yan et al., 2019; Bai et al., 2020). Additionally, the highest abundance of *Lactobacillus* was found in the XM2 group; strain XM2 isolated from native grass and the higher microbial populations in the XM2 group may be the main reason. *Weissella* was the other predominant microbe in all silages throughout the fermentation and was the early colonizer (Graf et al., 2016; Guan et al., 2018); *Pediococcus* contributed to an initial decline in silage pH, resulting in an anaerobic environment suitable for developing *Lactobacillus* (Yang et al., 2019). The previously published studies showed that *Weissella* was heterofermentative. These two LAB genera could not thrive at a pH environment lower than 4.5 and were thus active only during the early stages of ensiling. The follow-up lactic acid production mainly depends on *Lactobacillus*, which becomes active and thrives as pH decreases (Cai et al., 1998). However, the abundance of *Weissella* was significantly reduced in the 842 group, which could be explained by adding *L. graminis*, which could inhibit the growth of *Weissella*. The abundance of *Pediococcus* significantly increased in the 265 group, which could contribute to the addition of *P. acidilactici*. *Lactobacillus* was enriched in the XM2 group, and *Pediococcus* was enriched in the 265 group, which is in agreement with the addition of LAB in the XM2 and 265 groups.

CONCLUSION

The bacterial community of fresh native grass was discovered to be dominated by *Proteobacteria*, *Actinobacteriota,* and *Cyanobacteria*. This study demonstrated that the addition of LAB could influence silage fermentation by reconstructing microbiota. *Lactobacillus* was the dominant genus in the native grass silages, followed by *Enterobacter* and *Pediococcus*. Strain XM2 exhibited the potential possibility to respond to improving silage fermentation in native grass. Further results indicated that strain XM2 could effectively improve the silage quality, and it was proposed to be a potential starter culture for native grass silage.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SY: investigation, methodology, visualization, validation, data curation, writing—original draft, and conceptualization. SD: investigation, software, formal analysis, and writing—review and editing. GG: conceptualization, funding acquisition, supervision, and writing—review and editing. TW: project administration and supervision. YJ: conceptualization, funding acquisition, project administration, and supervision. All authors contributed intellectual input and assisted with this study and manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.731770/full#supplementary-material

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Top-Down Enrichment Strategy to Co-cultivate Lactic Acid and Lignocellulolytic Bacteria From the *Megathyrsus maximus* Phyllosphere

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Traditionally, starting inoculants have been applied to improve ensiling of forage used for livestock feed. Here, we aimed to build up a bioinoculant composed of lactic acid-producing and lignocellulolytic bacteria (LB) derived from the *Megathyrsus maximus* (guinea grass) phyllosphere. For this, the dilution-to-stimulation approach was used, including a sequential modification of the starting culture medium [Man, Rogosa, and Sharpe (MRS) broth] by addition of plant biomass (PB) and elimination of labile carbon sources. Along 10 growth-dilution steps (T1–T10), slight differences were observed in terms of bacterial diversity and composition. After the sixth subculture, the consortium started to degrade PB, decreasing its growth rate. The co-existence of *Enterobacteriales* (fast growers and highly abundance), *Actinomycetales*, *Bacillales*, and *Lactobacillales* species was observed at the end of the selection process. However, a significant structural change was noticed when the mixed consortium was cultivated in higher volume (500 ml) for 8 days, mainly increasing the proportion of *Paenibacillaceae* populations. Interestingly, *Actinomycetales*, *Bacillales*, and *Lactobacillales* respond positively to a pH decrease (4–5), suggesting a relevant role within a further silage process. Moreover, gene-centric metagenomic analysis showed an increase of (hemi)cellulose-degrading enzymes (HDEs) during the enrichment strategy. Reconstruction of metagenome-assembled genomes (MAGs) revealed that *Paenibacillus*, *Cellulosimicrobium*, and *Sphingomonas* appear as key (hemi)cellulolytic members (harboring endo-glucanases/xylanases, arabinofuranosidases, and esterases), whereas *Enterococcus* and *Cellulosimicrobium* have the potential to degrade oligosaccharides, metabolize xylose and might produce lactic acid through the phosphoketolase (PK) pathway. Based on this evidence, we conclude that our innovative top-down strategy enriched a unique bacterial consortium that could be useful in biotechnological applications, including the development/design of a synthetic bioinoculant to improve silage processes.

Keywords: bacterial consortia, lignocellulose, metagenome-assembled genomes, silage, bioinoculant, *Cellulosimicrobium*

INTRODUCTION

Ensiling is an ancient technique used to preserve nutrients in roughages (e.g., grass and legumes), generally offered as a ruminant livestock feed (Ávila and Carvalho, 2019). This process is based on spontaneous fermentation, where epiphytic lactic acid bacteria (LAB) metabolize plant-derived sugars, quickly decreasing the pH (between 5 and 4) and preventing silage spoilage (Fabiszewska et al., 2019). *Megathyrsus maximus* Jacq., a fast-growing, leafy perennial grass, is widely used in the tropics as roughage for livestock farming. Due to its high biomass availability (during both the dry and rainy seasons), protein content (~13%) and livestock digestibility (~60%), this grass has great potential for ensiling (Mojica-Rodríguez and Burbano-Erazo, 2020). However, *M. maximus* (guinea grass) like many tropical grasses contains a large amount of fiber, including lignin and structural carbohydrates linked to ferulic acid, preventing its efficient utilization directly in feedlots (Grabber et al., 2009). Ensiling of roughages comprises four phases: (1) microbial depletion of oxygen; (2) lactic acid production in anaerobic conditions (here LAB becomes highly abundant); (3) stabilization; and (4) feedout phase where silage is re-exposed to air (Keshri et al., 2019). The type of epiphytic microbiota depends on the forage crop, and is mainly composed of Gammaproteobacteria (e.g., *Klebsiella*, *Citrobacter*, *Pantoea*, and *Pseudomonas*), Firmicutes (e.g., *Clostridium*, *Bacillus*, and *Paenibacillus*), yeast, and molds, plus a low proportion of LAB (e.g., *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, and *Weissella*), actinomycetes and acetic/propionic acid bacteria (Nazar et al., 2021a,b). The phyllosphere of forage could represent an unexplored source of lignocellulolytic microbes that survive in oligotrophic conditions. Regarding ensiling, LAB populations are essential, but they do not exceed 1% of the total epiphytic microbiota (McAllister et al., 2018; Ávila and Carvalho, 2019; Fabiszewska et al., 2019). Therefore, the use of starting homofermentative LAB bioinoculants (e.g., *Lactobacillus* species) and other additives (e.g., organic acids and enzymes) has been used to improve the quality of the silage, thus benefiting animal productivity (Muck et al., 2018; Su et al., 2019). Additionally, (hemi)cellulases, amylases and proteases have also been added to forage at initiation of ensiling to improve the breakdown of plant polymers, providing labile sugars for lactic acid production and increasing the digestibility of plant cell walls (Windle et al., 2014; Addah et al., 2016; Ogunade et al., 2018). Moreover, it has been suggested that the inoculation of bacterial isolates and/or mixed microbial consortia (e.g., LAB plus lignocellulolytic species) with the potential to produce cellulases, arabinofuranosidases, xylosidases, ferulic acid esterases (FAE; EC 3.1.1.73), and cutinases, could enhance the quality of the silage by increasing the availability of carbohydrates with lower complexity (Muck et al., 2018; Tarraran and Mazzoli, 2018; Bonaldi et al., 2021). FAE has been recently reported in LAB, and its use may show a potential improvement on neutral detergent fiber (Xie et al., 2021).

The design and/or selection of microbial consortia with a desired structure/function can be accomplished by two strategies. In the top-down approach, microbial communities from nature are selected in a specific culture medium that enables the survival of the fittest in sequential transfers (Jiménez et al., 2014a; Díaz-García et al., 2021). On the other hand, in the bottom-up approach, the design of the consortium is carried out by mixing different populations of microbial isolates in specific proportions and compositions (Jiménez et al., 2018, 2020). Interestingly, a synthetic consortium composed of LAB and a cellulolytic fungus has been previously reported (Shahab et al., 2018). In this mutualistic guild, *Trichoderma reesei* provides the soluble saccharides (by depolymerization of plant polysaccharides), whereas *Lactobacillus pentosus* utilize them for the production of lactic and acetic acid. Based on these facts, we hypothesize that the selection of consortia composed of LAB and lignocellulolytic bacteria (LB), derived from the epiphytic community of forage biomass, and could be the starting point to design a synthetic bioinoculant that preserves the quality of the plant biomass (PB) during the ensiling process. In addition, the discovery of microbes or a set of mutualistic species that can produce lactic acid directly from PB is interesting from a biotechnological perspective (Tarraran and Mazzoli, 2018). Thus, in this study, we aimed to co-cultivate LAB and LB derived from the *M. maximus* cv. Agrosavia Sabanera phyllosphere by using a top-down approach (i.e., the dilution-to-stimulation strategy in which easy-to-consume carbon sources are eliminated and growth on the desired substrate is stimulated). During and after the selection process, different modifications of culture conditions were done. Additionally, bacterial 16S rRNA gene and whole metagenome sequencing, including the reconstruction of metagenome assembled genomes (MAGs), was performed to disentangle taxonomic and functional profiles of the obtained consortia. Based on this enrichment and metagenomic analysis, we report here the genomic capacity of a (hemi)cellulolytic *Cellulosimicrobium*-related species (an actinobacterium) to produce lactic acid from plant-derived xylose.

MATERIALS AND METHODS

Enrichment Strategy to Co-cultivated Lactic Acid and Lignocellulolytic Bacteria

The Guinea grass *M. maximus*-associated epiphytic microbial community was the source of inoculum for selection of LAB and LB populations. Firstly, this grass was cut in 1 cm^2 pieces. Then, in aseptic conditions, 10 fragments were inoculated in 10 ml of Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) and incubated at 39°C for 24 h. After microbial growth, 1 ml of the culture was inoculated in 9 ml of MRS broth and incubated to the above conditions. This latter procedure was carried out to pre-enrich LAB populations by growth of the community in medium designed to enrich growth of LAB. Subsequently, 25 µl of the pre-enriched culture were inoculated in 100 ml-flasks with 25 ml of MRS-modified broth (i.e., without glucose) containing 1% lignocellulosic substrate (*M. maximus* cv. Agrosavia Sabanera added as a carbon source; T1; **Figure 1**; **Table 1**), vitamin and trace elements solution (Jiménez et al., 2014b). Previously, the lignocellulosic substrate (supplied by Agrosavia) was knife-milled through a 1 mm screen

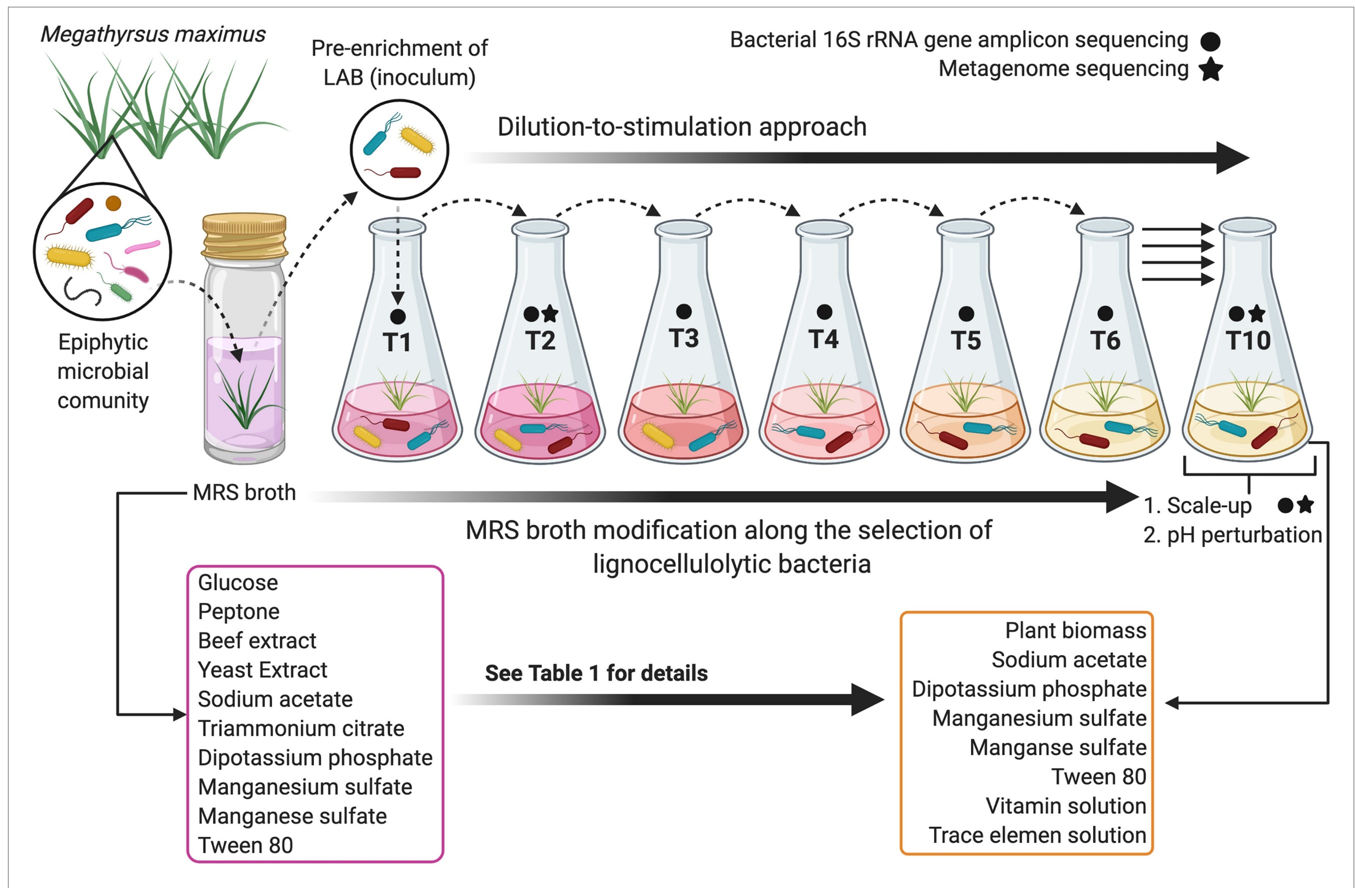

FIGURE 1 | Schematic representation of the top-down enrichment strategy by using the dilution-to-stimulation approach. During the selection process, the liquid Man, Rogosa, and Sharpe (MRS) medium, which is specific for lactic acid bacteria (LAB), was modified as shown in **Table 1** in order to select lignocellulolytic bacteria (LB) from the *Megathyrsus maximus* phyllosphere. The scale-up and pH perturbation were carried out after transfer 10 (T10). Created with BioRender.com.

TABLE 1 | Scheme of MRS broth modification during the enrichment strategy (from T1 to T6–T10).

MRS broth components (w/v)	Modification of MRS broth along the sequential transfers					
	T1	**T2**	**T3**	**T4**	**T5**	**T6–T10**
2% Glucose	1% PB	1% PB	1% PB	1% PB	1% PB	1% PB
1% Peptone	O	0.1%	0.01%	X	X	X
0.8% Beef extract	O	0.08%	0.008%	X	X	X
0.5% Sodium acetate	O	O	O	0.25%	0.25%	0.125%
0.4% Yeast extract	O	0.04%	0.004%	X	X	X
0.2% Triammonium citrate	O	O	O	0.1%	0.1%	X
0.2% Dipotassium phosphate	O	O	O	O	0.1%	0.1%
0.02% Magnesium sulfate	O	O	O	O	O	O
0.02% Manganese sulfate	O	O	O	O	O	O
45 µl/100 ml of Tween 80	O	O	O	O	22.5 µl/100 ml	22.5 µl/100 ml

The table shows the composition of modified medium for each enrichment step; all values are % (w/v) except for Tween 80 which is a volume ratio. (O) means the same percentage used in original MRS broth. (X) means the absence of this component within the modified culture medium. At T1, we replaced 2% glucose to 1% of PB and maintained it the same concentration in the subsequent transfers.

and washed twice with distilled water and ethanol 70% (v/v). The grass was collected at regrowth age of 30 days in a sub-region of the Colombian dry Caribbean (Motilonia RC, Cesar) with an elevation of 103 masl. The culture flasks were incubated at 28°C under shaking conditions (130 rpm). After 4 days of incubation, aliquots (25 µl) of microbial suspension were transferred to 25 ml of fresh liquid medium containing 1% plant biomass (T2), following the dilution-to-stimulation approach

(Jiménez et al., 2017). Two negative controls were also set up: (A) microbial inoculum in liquid modified MRS (without plant biomass and glucose) and (B) uninoculated liquid modified MRS (containing plant biomass and without glucose). During the growth-dilution transfers, liquid culture medium was sequentially modified (**Table 1**) to avoid the assimilation of labile carbon sources (e.g., ammonium citrate, yeast, and/or beef extract), increasing the selection for LB populations. Thus, these modifications were done until no growth in negative control A was observed (**Figure 1**; **Table 1**). At the end of each transfer cultures were filtered and the remain substrate was dried for 24 h at 45°C to calculate the percentage of weight loss, following the formula proposed by de Lima Brossi et al. (2016). Bacterial growth was determined using optical density (OD) at 600 nm and the number of viable cells (CFU/ml) was quantified by plate counting in R2A and MRS agar (Merck, Darmstadt, Germany). Moreover, samples from transfer 10 (T10) were conserved in 20% of glycerol at −20°C. ANOVA and *post hoc* Tukey-Kramer test were performed using the software R (R Core Team, 2008) to evaluate the significant difference in substrate consumption and bacterial growth along the transfers, with a CI of 99% ($\alpha = 0.01$).

Growth of the Bacterial Consortium at Different Volume and pH Values

To increase the bacterial biomass, the mixed culture after T10 was transferred to a final volume of 500 ml. We carried out this procedure because this consortium was used as a starting bioinoculant in a silage of tropical forages in field experiments (data not shown). The scale-up growth process began with a pre-inoculum from T10 that was cultivated in 25 ml of liquid medium (eight replicates), as previously described. Two of these replicates were mixed to produce an inoculum of 50 ml. To ensure a high bacterial density, four 2 L-flasks with 500 ml of fresh medium (same composition as T10) were inoculated with the former 50 ml and incubated at 28°C for 8 days. Finally, four replicates were mixed and filtered to produce 2 L of bacterial culture. Moreover, the consortium obtained at T10 was cultivated (i.e., perturbed) on the modified MRS medium adjusted to different pH values (4, 5, and unmodified pH 6.2) at 28°C for 4 days, with shaking at 130 rpm (in two sequential transfers). Number of viable cells (CFU/ml) was quantified by plate counting in R2A and MRS agar (Merck, Darmstadt, Germany). OD at 600 nm and weight loss of the substrate were calculated with the parameters described above. After microbial growth in different pH values, the secreted fractions of the cultures were tested for the presence of plant polymers-degrading endo-enzymes using a set of six chromogenic polysaccharide hydrogels (CPH; i.e., 2-HE-cellulose, carboxymethylcellulose, arabinoxylan, xylan, xyloglucan, and glucomannan) and two insoluble biomass substrates (ICB; i.e., wheat straw and sugarcane bagasse; Kračun et al., 2015). The semi-quantification of the enzymatic activities was measured as reported Díaz-García et al. (2021) and following the instructions of the manufacturer (GlycoSpot IVS, Farum, Denmark). ANOVA and *post hoc* Tukey-Kramer test were performed using the software R (R Core Team, 2008) to evaluate significant differences in growth and enzyme activities, with a CI of 99% ($\alpha = 0.01$).

Total DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Total microbial DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. For the 16S rRNA gene analysis, triplicate samples from the dilution-to-stimulation approach (T1, T2, T3, T4, T5, T6, and T10), after the scale-up process (S) and the pH perturbation (4, 5, and unmodified) were sequenced using the Illumina MiSeq technology (300 bp pair-end reads). For each sample, a library of 16S rRNA gene amplicons (hypervariable region V3–V4) was prepared using the primers 341F and 785R (Klindworth et al., 2013). Bioinformatic analysis was done using the software Quantitative Insights Into Microbial Ecology (QIIME2; Bolyen et al., 2019). VSEARCH (Rognes et al., 2016) was used to join raw pair-end reads. Quality control and read correction to obtain amplicon sequence variants (ASVs) were done using Deblur pipeline (Amir et al., 2017). Rarefaction was performed (5,205 and 5,490 reads for the enrichment steps and the pH perturbation samples, respectively) per sample to calculate alpha and beta diversity (including Shannon's index and weighted UniFrac distances), and for comparisons between samples (e.g., relative abundance of ASVs). MAFFT (Katoh and Standley, 2013) was used to construct multiple alignments of ASVs. A phylogenetic tree was built using FasTree (Price et al., 2009) based on MAFFT alignment. Taxonomic affiliation of ASVs was done using SILVA rRNA gene curated database (Quast et al., 2013).

Metagenome Sequencing and Gene-Centric Analysis

Based on the 16S rRNA sequencing results, consortia T2, T10, and S (in duplicate) were subjected to Illumina MiSeq technology sequencing (300 bp paired-end reads) in order to determine taxonomy profiles and metabolic potential. After whole-metagenome sequencing, the FastQ files were uploaded in the MG-RAST server (Meyer et al., 2008). Overlapping sequence pairs were matched, and non-overlapping reads retained as individual reads, after which, dereplication was performed. Duplicate read-based inferred SE estimation and quality trimming (phred score < 20) used default settings. Gene predictions were done using the FragGeneScan software, and subsequently, the proteins were annotated based on BLASTX searches against the RefSeq and KEGG databases using an *e*-value cutoff of 1e-15, a minimum alignment length of 50 amino acids, and a minimum identity of 50% (Jiménez et al., 2016). Data from MG-RAST annotation were statistically analyzed using the STAMP package (Parks and Beiko, 2010). Moreover, to evaluate the relative abundance of reads per selected enzyme-encoding gene, the counts were normalized to hits, or unique matches, per million reads, thereby accounting for differences in metagenome sizes. Nine genes involved in heterolactic and/or homolactic fermentation of xylose (Tarraran and Mazzoli, 2018)

and 10 genes involved in lignocellulose degradation (Jiménez et al., 2014a; Díaz-García et al., 2021) were analyzed. Heat maps were constructed in the web server Heatmapper using row-Z score for each enzyme (Babicki et al., 2016).

Reconstruction and Analysis of Metagenome-Assembled Genomes

For the assembly of MAGs, raw sequence data (FastQ files) from T2, T10, and S samples were initially trimmed using the Sickle tool v1.33 with default parameters (available at https://github.com/najoshi/sickle). To avoid misleading results from subsequent binning analysis, carp artifacts were detected. For this, trimmed sequences were filtered against adapter and non-authentic primer sequences originating from the Illumina library preparation (Marter et al., 2021). Metagenome assembly was done using MEGAHIT v1.2.7 (Li et al., 2015) with default parameters. Concoct v1.1.0 (Alneberg et al., 2014), Bowtie v2.3.5 (Langmead and Salzberg, 2012), MetaBAT v2.12.1 (Kang et al., 2015), and MaxBin v2.2.6 (Wu et al., 2016) were used to bin the assembled sequences with default parameters. Binned contigs obtained were subsequently analyzed using DAS tool v1.1.2 (Sieber et al., 2018). To assess the completeness and contamination of the resulting bins, CheckM v1.0.13 was used with the lineage_wf option (Parks et al., 2015). The MAGs were structurally annotated and curated based on rRNAs genes (e.g., 5S, 16S, and 23S) and *rpoB* gene using DFAST v1.2.6 (Tanizawa et al., 2018). Taxonomic assignment was performed using GTDB v1.4.1 (Parks et al., 2020). Functional annotation was done using the RAST (Aziz et al., 2008) and dbCAN webservers (Yin et al., 2012). Moreover, 24 encoding-genes involved in hetero- or homo-fermentation of pentoses and hexoses (Hatti-Kaul et al., 2018; Tarraran and Mazzoli, 2018) were searched within the MAGs.

RESULTS

Enrichment Strategy to Co-cultivate Lactic Acid and Lignocellulolytic Bacteria

The selection of consortia was carried out by the dilution-to-stimulation approach using the epiphytic microbial community of *M. maximus* as starting inoculum (**Figure 1**). In the first stage of selection, we aimed to increase the populations of LAB by growing the phyllosphere-derived community in MRS broth. This pre-enrichment was used to inoculate the first transfer (T1), where glucose was removed and 1% (w/v) *M. maximus* was added as a carbon source. After this stage, increase of LB populations was the main goal. For this purpose, liquid MRS medium was sequentially modified from T2 to T6–T10, removing peptone, yeast extract, beef extract, triammonium citrate, and decreasing the concentration of sodium acetate, dipotassium phosphate, and tween 80 (**Table 1**). These modifications were carried out until microbial cell growth (measured by OD at 600 nm) in the negative control (i.e., without plant biomass) was not observed, indicating that the consortium started to consume the plant polymers (**Figure 2A**). After T6, the selected consortium was able to consume ~14% of plant biomass and significant differences (value of $p < 0.01$) in this capacity were evidenced compared to T5 (**Figure 2B**). Moreover, progressive reduction of bacterial growth rate was observed from T1 to T6, suggesting that labile carbon sources were depleted due to the liquid medium modification (**Figures 2A,C**). LAB populations were abundant at T1–T2. However, they decreased along the enrichment strategy (**Figure 2D**). At the end of the selective process (T10), around 10^9 and 10^5 CFU/ml of total and LAB populations were obtained, respectively (**Figures 2C,D**).

Bacterial Diversity and Composition Along the Enrichment Strategy

The bacterial community changes during the selective process were evaluated by 16S rRNA gene sequencing. In total, this analysis generated 1,623 ASVs and ~1.2 Gbp of high-quality reads. The results indicated that bacterial diversity slightly decreased from T1 to T5. However, the number of observed ASVs increased from T5 (44 ± 3.0) to T6 (77 ± 4.1) and T10 (73 ± 4.5). Interestingly, after the scale-up process (to 500 ml of culture volume) the number of ASVs increased to 99 ± 4.0 (**Figure 3A**). The taxonomic affiliation of the 16S rRNA data revealed that *Gammaproteobacteria* and *Bacillales* species were mostly selected during the enrichment strategy. Regarding LAB populations, species belonging to the *Enterococcaceae* family were observed at T1–T2 (~10% of the total community; **Figure 3B**). They remained in low abundance in the final selected consortium (i.e., T10). Modifications in the MRS liquid medium did not greatly affect the bacterial structural composition between T2 and T10. Remarkably, the final consortium (i.e., T10) showed a significant change after its cultivation in a higher volume (i.e., 500 ml; **Figure 3C**). This scale-up process (denominated S in **Figure 3**) mostly increased the relative abundance of *Paenibacillaceae*, *Sphingomonadaceae*, and *Xanthobactereaceae*, well known lignocellulolytic families (**Figure 3B**). At the end, the mixed bioinoculant (S), used in the field silage experiment, contained a higher proportion of LB populations and lower proportion of LAB (mostly species from *Enterococcaceae* family).

Gene-Centric Metagenomic Analysis: Taxonomy and Function Within Consortia

The mixed consortia obtained at T2, T10, and S was subjected to a whole-metagenomic sequencing and analysis. Approximately 1.3, 0.9, and 3.0 Gbp were obtained in T2, T10, and S, respectively, with a read average size of 300 bp. Based on the taxonomic affiliation of reads, four bacterial classes were highly abundant. In this regard, *Enterobacteriales* and *Lactobacillales* decreased from T2 to T10 and S, while *Actinomycetales* and *Bacillales* increased from T2 to T10 and S (**Figure 4A**). *Enterobacteriales* species were the predominant taxa (between 50 and 90% of total affiliated sequences) within consortia. At T10 and S, the relative abundance of *Lactobacillales* was lower than at T2, decreasing from about 4% to between 1 and 3% (**Figure 4A**). Regarding specific members of LAB, *Enterococcus* sp. was the

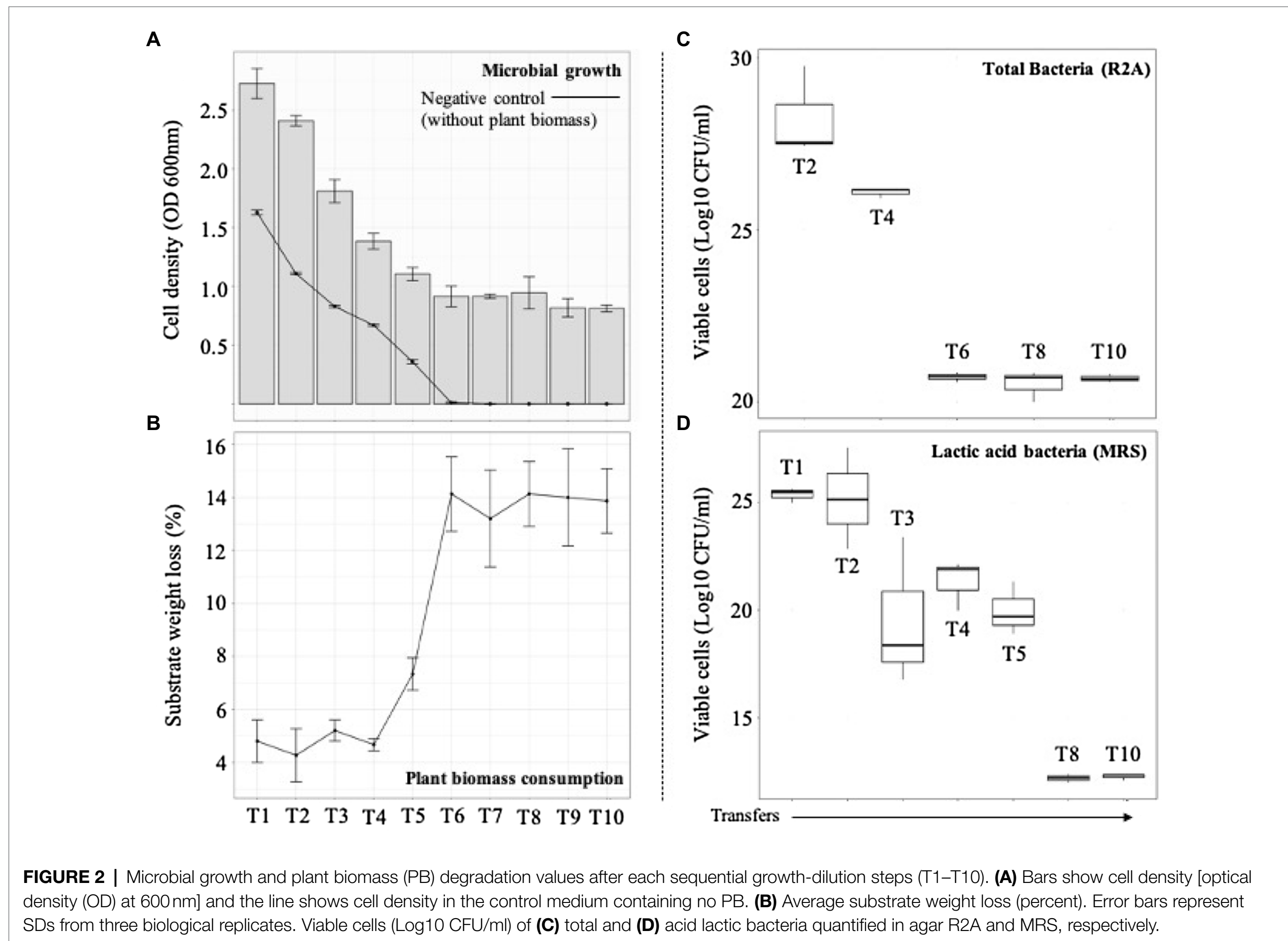

FIGURE 2 | Microbial growth and plant biomass (PB) degradation values after each sequential growth-dilution steps (T1–T10). **(A)** Bars show cell density [optical density (OD) at 600 nm] and the line shows cell density in the control medium containing no PB. **(B)** Average substrate weight loss (percent). Error bars represent SDs from three biological replicates. Viable cells (Log10 CFU/ml) of **(C)** total and **(D)** acid lactic bacteria quantified in agar R2A and MRS, respectively.

most abundant genus (between 1 and 5% relative abundance) in T2, T10, and S. Other genera, like *Lactobacillus* sp., *Leuconstoc* sp., *Pediococcus* sp., and *Weissella* sp. were found in very low abundance (less than 0.15%) in all the stages of selection, although their populations increased in S with respect to T10 (**Supplementary Figure S1**). Regarding fungal communities, metagenome data revealed that they are found in very low relative abundance (less than 0.001%). Based on the function (KO level), consortia T2 and T10 showed a similar profile, compared with the consortium obtained after scale-up (S) process (**Figure 4B**). Moreover, the results showed that nine of 10 genes involved in lignocellulose transformation were enriched from T2 to T10 and S (**Figure 4C**). Regarding genes relevant to lactic acid production, we observed that genes encoding xylose dissimilation activities [e.g., transketolase (TK) and transaldolase (TA)] were highly abundant at T2, whereas others [e.g., 6-phosphofructokinase and L-lactate dehydrogenase (LDH)] were highly abundant at T10 and S (**Figure 4C**).

Response of the Mixed Microbial Consortium in a pH Gradient

The ability of the selected consortium (i.e., T10) to grow in acidic silage conditions was evaluated by modifying the initial pH of the liquid medium. At pH 4, a significant (value of $p < 0.01$) increase of LAB was evidenced, showing a viable population density of around 4×10^8 CFU/ml. In contrast, total bacterial cells decreased at pH 4 and pH 5, compared to the unmodified liquid medium (pH around 6.2; **Figure 5A**). After the perturbation at different pH values, the metasecretome of the consortium was evaluated for the presence of plant polymer-degrading endo-enzymes. The highest and significant (value of $p < 0.01$) enzymatic activities on a set of CPHs and ICBs were obtained in the metasecretome of the mixed consortium after growth at pH 5 (**Figure 5B**; **Supplementary Figure S2**). At this pH, the plant biomass consumption was 17.8 ± 0.6%. Additionally, 16S rRNA gene sequencing analysis were carried out after the perturbation at different pH values. On average, 104 ASVs were generated from 50 Mbp of high-quality reads. Alpha diversity results demonstrated a decrease of observed ASVs in the pH gradient, reaching a value of 31 ± 1.0 at pH 4 (**Figure 5C**). Interestingly, a significant (value of $p < 0.001$) increase in the relative abundance of *Actinomycetales* and *Lactobacillales* orders was observed at pH 5 (**Figure 5D**). However, the highest abundant order was *Enterobacteriales* (**Supplementary Figure S3**).

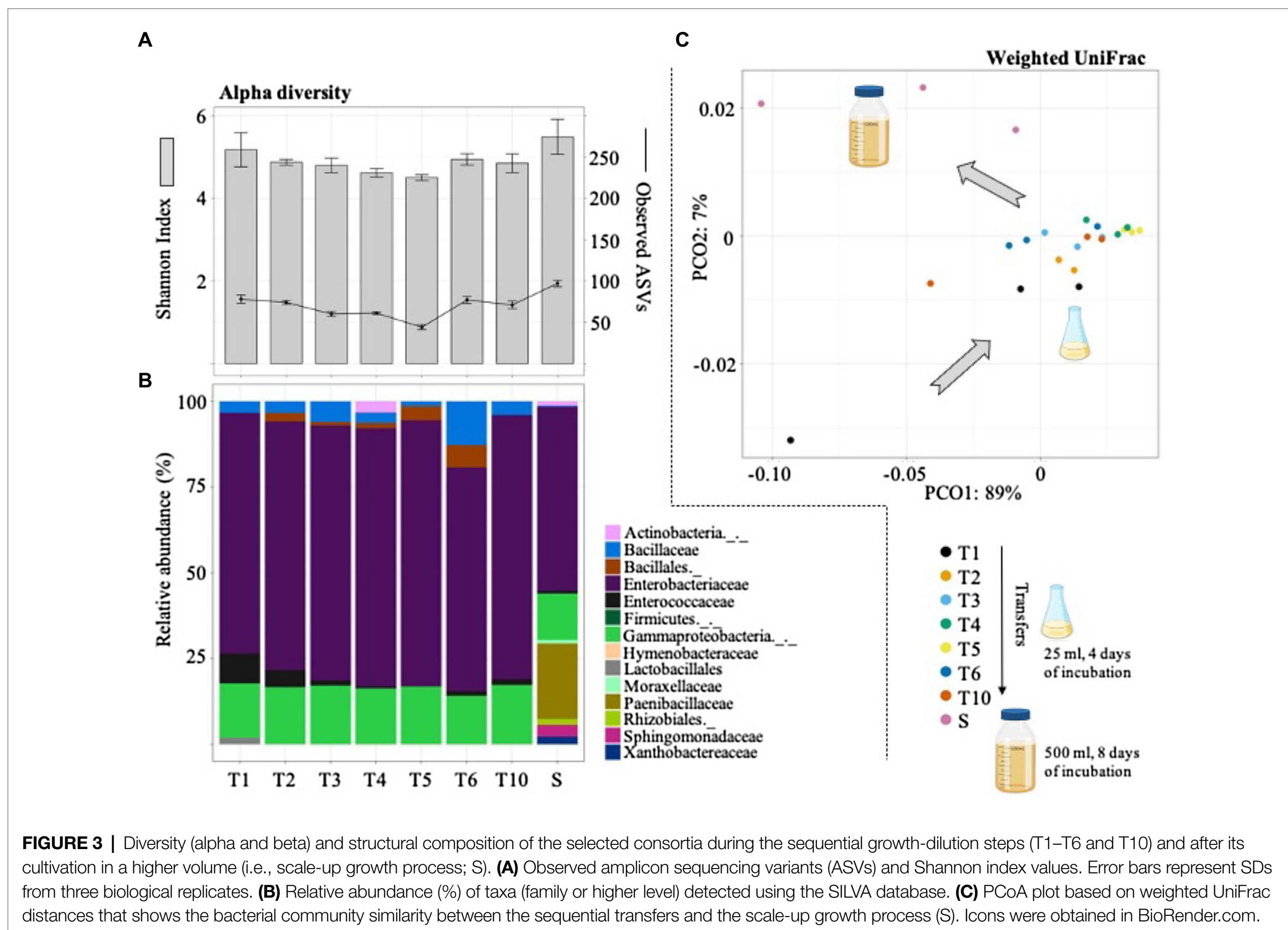

FIGURE 3 | Diversity (alpha and beta) and structural composition of the selected consortia during the sequential growth-dilution steps (T1–T6 and T10) and after its cultivation in a higher volume (i.e., scale-up growth process; S). **(A)** Observed amplicon sequencing variants (ASVs) and Shannon index values. Error bars represent SDs from three biological replicates. **(B)** Relative abundance (%) of taxa (family or higher level) detected using the SILVA database. **(C)** PCoA plot based on weighted UniFrac distances that shows the bacterial community similarity between the sequential transfers and the scale-up growth process (S). Icons were obtained in BioRender.com.

Metagenome-Assembled Genomes Obtained From the Mixed Consortia

A total of 2, 3, and 9 high quality MAGs (>90% of completeness and <3.5% of contamination) were reconstructed from the T2, T10, and S metagenomes, respectively (**Table 2**; **Supplementary Table S1**). The genome lengths ranged from 2.28 to 5.93 Mb. At T2 and T10, MAGs belong to *Cellulosimicrobium funkei* (Bin7_T2 and Bin8_T10) and *Klebsiella aerogenes* (Bin25_T2 and Bin2_T10) were obtained. Interestingly, the data revealed that three different MAGs from *Enterococcus* species were obtained. At T2, a single genome associated to *Enterococcus faecalis*, while in both T10 and S, two genomes associated to *Enterococcus casseliflavus*. Functional annotation of all reconstructed MAGs showed that Bin7_T2, Bin8_T10, Bin3_T10, Bin11_S, Bin6_S, Bin002_S, and Bin8_S contain more than nine genes-encoding glycosyl hydrolases (GHs) from CAZy families involved in degradation of (hemi)cellulose (**Table 2**). Moreover, Bin3_T10 and Bin6_S affiliated to *E. casseliflavus* harbor more than 20 GHs involved in transformation of oligosaccharides (e.g., GH1, GH2, and GH3). In addition, two LDHs encoding-genes were found in those two MAGs (**Table 2**; **Supplementary Table S2**).

Analysis of MAGs With the Potential to Produce Lactic Acid

A total of five MAGs (Bin6_S, Bin002_S, Bin2_S, Bin4_T10, and Bin44_S) were selected in order to analyze their potential to produce lactic acid from sugars. These MAGs contained at least one LDH encoding-gene (**Table 2**). Based on the functional annotation using the RAST server, encoding-genes involved in fermentation of pentoses and hexoses were detected within these five MAGs (**Supplementary Table S2**). The results showed that Bin6_S (*E. casseliflavus*), Bin002_S (*C. funkei*), and Bin2_S (*K. aerogenes*) encode complete enzymatic machinery to produce lactic acid. Specifically, Bin6_S and Bin002_S contain the genes necessary for heterolactic acid fermentation of pentoses (e.g., xylose and arabinose) through the phosphoketolase (PK) pathway (**Figure 6**). In addition, Bin002_S has the metabolic potential to carry out a homolactic fermentation of xylose through the pentose phosphate (PP) pathway (**Figure 6**) and heterolactic fermentation of maltose through the PK pathway. Moreover, Bin2_S contains the gene capability to produce D-lactate from glycerol through an Embden–Meyerhof (EM) glycolytic pathway (**Supplementary Table S2**).

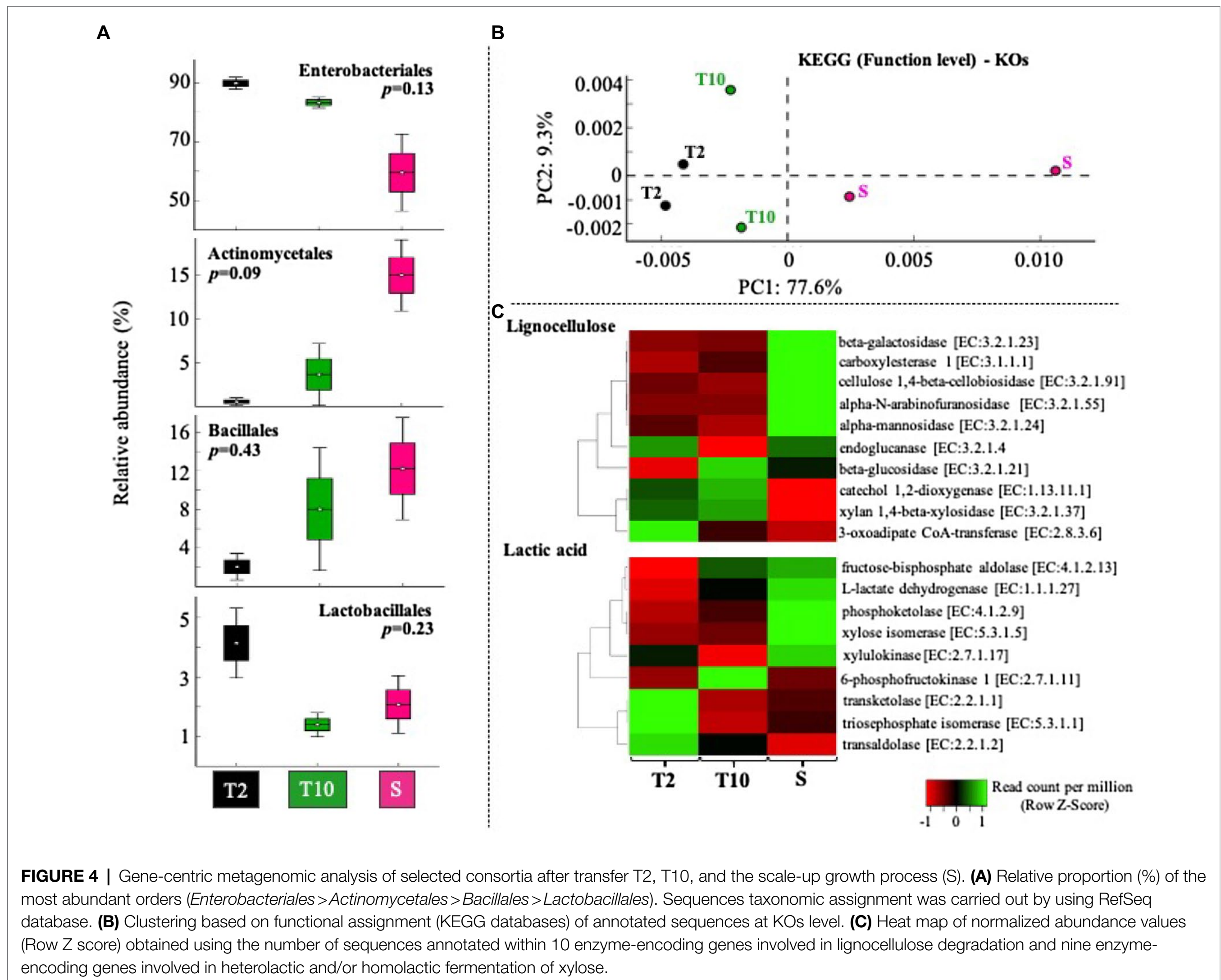

FIGURE 4 | Gene-centric metagenomic analysis of selected consortia after transfer T2, T10, and the scale-up growth process (S). **(A)** Relative proportion (%) of the most abundant orders (*Enterobacteriales > Actinomycetales > Bacillales > Lactobacillales*). Sequences taxonomic assignment was carried out by using RefSeq database. **(B)** Clustering based on functional assignment (KEGG databases) of annotated sequences at KOs level. **(C)** Heat map of normalized abundance values (Row Z score) obtained using the number of sequences annotated within 10 enzyme-encoding genes involved in lignocellulose degradation and nine enzyme-encoding genes involved in heterolactic and/or homolactic fermentation of xylose.

DISCUSSION

The ensiling of forage crops is a relevant process that enhances feed quality and productivity in livestock systems. The design, application, and testing of starting inoculants is a world-wide priority of research within this field (Muck et al., 2018; Fabiszewska et al., 2019; Nazar et al., 2021a). Thus, this study aimed to build up a unique bioinoculant composed of LAB and LB derived from the phyllosphere of *M. maximum* cv. Agrosavia Sabanera. This Guinea grass is recognized as one of the best species that improves beef and milk production, especially in tropical countries (Villegas et al., 2020; Carvajal-Tapia et al., 2021). In addition, this pasture crop has an excellent nutritional quality and the ability to tolerate adverse abiotic conditions (e.g., drought), being a species of interest for arid regions (Benabderrahim and Elfalleh, 2021). However, the information about its epiphytic microbial community as a source of LAB and LB is null. Ruminal-derived microbial communities could contain higher proportion of LAB and LB. However, we have selected roughage phyllosphere because its microbial communities are the starter inoculant in a silage process.

Previously, the design of consortia composed of lactic acid-producing and lignocellulolytic microorganisms has been achieved by the co-culture of axenic strains (Li et al., 2018; Shahab et al., 2018). In the current work, we developed an innovative top-down strategy to co-cultivate LAB and LB populations using a rational and sequential modification of MRS broth throughout the dilution-to-stimulation approach (**Figure 1**; **Table 1**). During the enrichment process, a high abundance of species from *Enterobacteriaceae* family was noticed. Within this enriched system, they could take advantage due to their evolutionary adaptation to the *M. maximum* phyllosphere and also the capacity to grow quickly in liquid environments with labile and plant-derived carbon sources (e.g., glucose; Goldford et al., 2018; Díaz-García et al., 2021). We hypothesized that *Enterobacteriaceae* species could compete with LAB and LB for the uptake of oligo or monosaccharides, having a

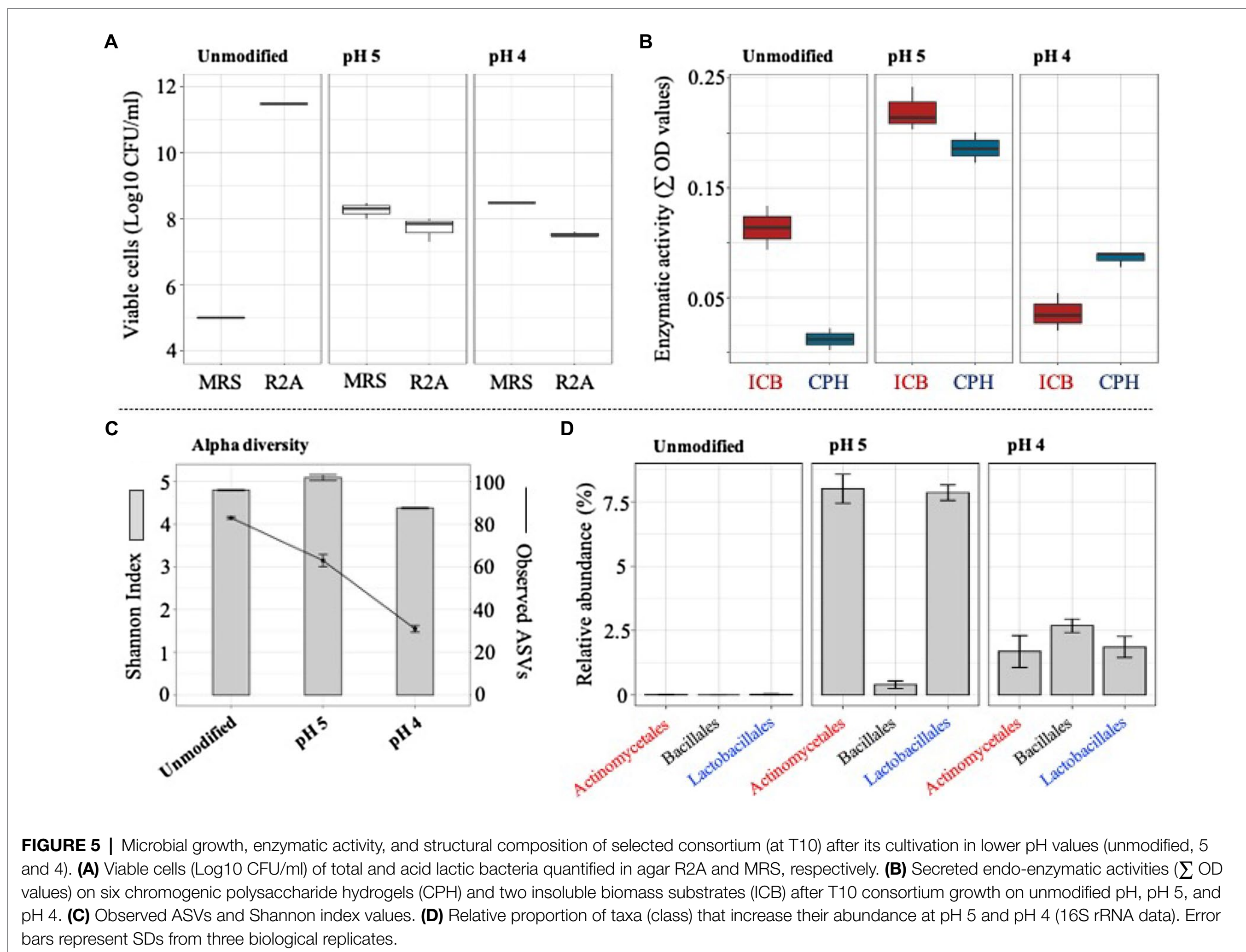

FIGURE 5 | Microbial growth, enzymatic activity, and structural composition of selected consortium (at T10) after its cultivation in lower pH values (unmodified, 5 and 4). **(A)** Viable cells (Log10 CFU/ml) of total and acid lactic bacteria quantified in agar R2A and MRS, respectively. **(B)** Secreted endo-enzymatic activities (∑ OD values) on six chromogenic polysaccharide hydrogels (CPH) and two insoluble biomass substrates (ICB) after T10 consortium growth on unmodified pH, pH 5, and pH 4. **(C)** Observed ASVs and Shannon index values. **(D)** Relative proportion of taxa (class) that increase their abundance at pH 5 and pH 4 (16S rRNA data). Error bars represent SDs from three biological replicates.

secondary role in the deconstruction of lignocellulose, where they could be considered as sugar cheaters (Jiménez et al., 2017). In silage, the proliferation of *Enterobacteriaceae* species is undesirable because they compete with LAB, can degrade proteins to produce biogenic amines and branched fatty acids (Ávila and Carvalho, 2019).

Although, the modification of MRS broth did not significantly affect the bacterial composition on a taxonomic basis, the substrate weight loss values (after T6) and the enrichment of some enzyme encoding-genes (i.e., from T2 to T10 and S) suggested that these changes were effective to increase the proportion of LB. However, at the end of the selective process (i.e., T10), a co-existence of LAB (mostly belonged to *Lactobacillales*) and LB (e.g., *Actinomycetales* and *Bacillales*) was observed. In silage, we hypothesized that the action of lignocellulolytic enzymes could improves plant polysaccharides degradation, proving fermentable sugars for the entire microbial community, including LAB. In this regard, the supplementation of cellulases and LAB has shown positive effects in the silage of tropical grasses (Khota et al., 2016). In our enrichment strategy, LAB can grow more slowly because they are metabolically less efficient, but they are competitive and can thrive as a minor fraction of the population with an important ecological role by employing several mechanisms to inhibit the growth of other species (e.g., production of organic acids and hydrogen peroxide; Ávila and Carvalho, 2019). Other mechanism that could back up the coexistence of LB and LAB is the cross-feeding. In this scenario, LB can growth using plant-derived monosaccharides, secreting molecules that LAB might consume (Seth and Taga, 2014).

Many factors, such as nutrient availability, biological interactions, temperature and pH modulate population dynamics in microbial systems (Ratzke and Gore, 2018; Xu et al., 2020; Estrela et al., 2021). In this study, drastic changes in the structure/function of the final selected consortium (i.e., T10) were observed after its growth in a higher volume (500 ml) and longer time of incubation (8 days; i.e., the scale up growth process). We hypothesized that incubation time, oxygen availability, nutrient content/exchange, and spatial co-localization of the bacterial consortium members (in above mentioned culture conditions) could be factors that drive the populations dynamics in this system (Takahashi and Aoyagi, 2018;

TABLE 2 | Features of metagenome assembled genome (MAGs) reconstructed from T2, T10, and S consortia.

Transfer	Bin ID	Completeness (%)	Contamination (%)	# contigs	Genome size (Mb)	Probable taxa*	Total # CAZymes	ODE (GHs)	HDE (GHs)	CE1	LDHs
T2	Bin7_T2	80.35	1.45	905	3.42	*Cellulosimicrobium funkei*	75	11	10	1	2
T2	Bin2_T2	99.63	0.37	167	2.78	*Enterococcus faecalis*	114	11	0	0	2
T2	Bin25_T2	98.5	2.33	186	5.14	*Klebsiella aerogenes*	119	12	3	2	2
T10	Bin3_T10	71.84	0.31	733	2.28	*Enterococcus casseliflavus*	119	26	14	0	2
T10	Bin8_T10	98.84	1.25	327	4.32	*Cellulosimicrobium funkei*	131	12	10	2	2
T10	Bin4_T10	92.82	0.85	1,006	4.71	*Bacillus paranthracis*	80	2	0	0	2
T10	Bin2_T10	99.92	1.2	101	5.05	*Klebsiella aerogenes*	120	12	3	0	2
S	Bin37_S	95.11	2.62	313	3.06	*Aeromicrobium camelliae*	45	0	1	0	0
S	Bin15_S	98.83	0.34	36	5.06	*Tardiphaga* sp.	79	2	0	1	0
S	Bin11_S	97.84	0.28	143	3.78	*Sphingomonas* sp.	101	10	15	1	0
S	Bin44_S	89.15	0.91	574	4.05	*Aureimonas* sp.	52	0	0	1	1
S	Bin4_S	91.47	6.2	896	4.35	*Brevibacillus borstelensis*	41	1	0	0	0
S	Bin6_S	98.4	3.11	262	3.32	*Enterococcus casseliflavus*	154	34	16	1	2
S	Bin18_S	98.64	2.03	96	5.73	*Brevibacillus* sp.	62	2	1	0	0
S	Bin2_S	94.9	0.2	113	4.82	*Klebsiella aerogenes*	109	12	3	0	2
S	Bin002_S	99.42	2.41	131	4.41	*Cellulosimicrobium funkei*	131	12	10	2	2
S	Bin8_S	98.84	0.23	184	5.93	*Paenibacillus nanensis*	242	24	34	0	0

*Taxa were assigned based on rpoB blast and average nucleotide identity criteria (Parks et al., 2020).
ODE, oligosaccharide-degrading enzymes; HDE, (hemi)cellulose-degrading enzymes; GHs, glycosyl hydrolases; CE, carbohydrate esterases; and LDHs, lactate dehydrogenases (EC 1.1.1.27 and EC 1.1.1.28).

Jawed et al., 2019; van Tatenhove-Pel et al., 2021). In addition, these results suggested that our mixed consortium, composed of around 70 bacterial species, is still unstable. A microbial inoculant with less diversity could be more stable (Jiménez et al., 2017). After the scale up growth process, an increase of species from *Paenibacillaceae* family was observed. These types of bacteria could have lower growth rates in a competitive environment and dominate later stages of carbon decomposition (Zhang et al., 2020; Díaz-García et al., 2021). Within silage, *Paenibacillus* species (spore-forming bacteria) could enhance growth of LAB, tolerate acidic conditions and produce bacteriocins that inhibit yeast and molds (Xu et al., 2018; Singh et al., 2019). However, it is important to note that in some cases *Paenibacillus* can compete with LAB for fermentable sugars, limiting its growth (Li et al., 2020). In addition, they have a vast lignocellulolytic potential that may improve the digestion of complex carbohydrates in the bovine rumen (Ticona et al., 2021). *Paenibacillus* species can survive harsh environmental conditions and can be detected even in the last stages of ensiling (Ning et al., 2017). On the other hand, the pH has also modulated the structure, diversity, and metabolic activity of the selected consortium (i.e., T10). In this regard, an increase of lignocellulose consumption and enzymatic activities was observed at pH 5. These results were correlated with a significant increase of *Actinomycetales* and *Lactobacillales* species (**Figure 5**), suggesting that these taxa could be involved in plant biomass transformation and might be metabolically active in a further ensiling process. It has been reported that species from the phylum *Actinobacteria* are highly abundant in fresh forages (e.g., alfalfa, barley, triticale, and oat), but decrease in abundance during the ensiling processes (Duniere et al., 2017; Hu et al., 2020). Low abundance of *Actinobacteria* and high abundance of *Protobacteria* phylum has been observed in Napier grass silage (Nazar et al., 2021a). Species from *Actinobacteria* generally do not grow below pH 4.5 under microaerophilic conditions, and therefore its growth is unlikely to occur when the pH of the silo stabilizes (Hill et al., 2001). Thus, we presumed that *Actinomycetales* with the capacity to thrive at lower pH values were selected in our mixed consortium.

Based on MAGs annotation, *Paenibacillus*, *Cellulosimicrobium*, and *Sphingomonas* appear as key (hemi)cellulolytic members, while *Cellulosimicrobium*, *Enterococcus*, and *Klebsiella* are the lactic acid producers within the mixed consortium. Their coexistence can be related to the preferential metabolism of different plant-derived hexoses (i.e., glucose or galactose) and pentoses (i.e., xylose and arabinose; Hatti-Kaul et al., 2018). Within the mixed consortium, *Enterococcus* species have the potential to transform xylooligosaccharides and/or arabinoxylans. Although rare, the presence of GH1, GH2, GH3, GH5, GH30, GH43, and GH67 families were found in Bin6_S and Bin3_T10. This information could add new perspectives on the carbohydrate metabolism of LAB species involved in the fermentation of hemicellulose-containing substrates (Michlmayr and Kneifel, 2014). To highlight, we found that *C. funkei* (Bin002_S) has the genomic potential to produce lactic acid from xylose and maltose (through the PK and PP metabolic pathways). Two different pathways are proposed for pentose metabolism, the

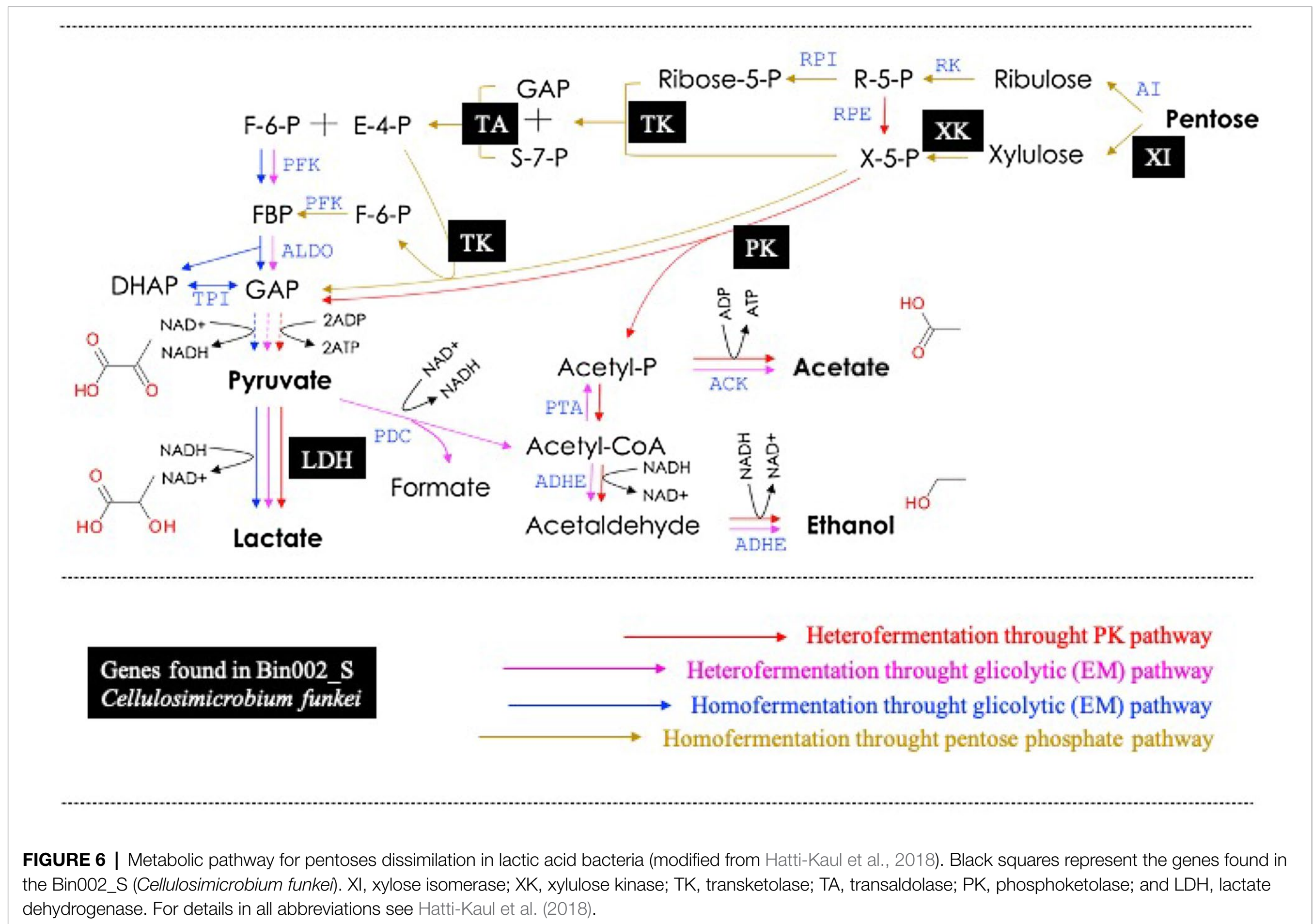

FIGURE 6 | Metabolic pathway for pentoses dissimilation in lactic acid bacteria (modified from Hatti-Kaul et al., 2018). Black squares represent the genes found in the Bin002_S (*Cellulosimicrobium funkei*). XI, xylose isomerase; XK, xylulose kinase; TK, transketolase; TA, transaldolase; PK, phosphoketolase; and LDH, lactate dehydrogenase. For details in all abbreviations see Hatti-Kaul et al. (2018).

PK pathway yields 1 mol lactic acid/mol sugar, whereas the pentose phosphate pathway provides a theoretical lactic acid yield of 1.67 mol/mol sugar (Tanaka et al., 2002). Based on the CAZyme annotation, *C. funkei* could produce enzymes involved in (hemi)cellulose degradation (e.g., GH16, GH30, GH43, and GH51; Ventorino et al., 2015; Dou et al., 2020). To the best of our knowledge, no native (hemi)cellulolytic LAB from *Actinobacteria* phylum has been previously reported. Interestingly, we also found that Bin002_S contained two carbohydrate esterase (CE1) enzymes that may improve the digestibility of forage within the rumen (Li et al., 2021). However, the presence of FAE was not detected within the reconstructed MAGs. Interestingly, *Cellulosimicrobium* and *Paenibacillus* species have been cataloged as desirable microorganisms in cassava foliage silage (Li et al., 2020). Finally, this study reported the design and characterization of a mixed consortium, obtained from the *M. maximus* phyllosphere, composed of LAB and LB. Based on the results, we conclude that a synthetic bioinoculant composed of strains of *Cellulosimicrobium*, *Enterococcus*, and *Paenibacillus* species could be used to improve the quality of silage processes. Further studies with our mixed consortium will: (i) apply it as starting inoculant in an ensiling process and evaluate the consequences in terms of quality and the dynamics of microbial populations; (ii) isolate the key bacterial members; (iii) design a synthetic consortium using bacterial axenic cultures (bottom-up approach) and test it in an ensiling process; and (iv) evaluate lactic acid production by *Cellulosimicrobium via* genome-based metabolic reconstruction using Bin002_S.

DATA AVAILABILITY STATEMENT

The bacterial 16S rRNA gene amplicon sequencing data obtained in this study have been deposited under NCBI BioProject accession number PRJNA734654. All metagenome sequences are publicly accessible on the MG-RAST server (Metagenome IDs mgm4912842.3 to mgm4912847.3).

AUTHOR CONTRIBUTIONS

LD-G carried out all wet-lab experiments and bioinformatic analysis and helped to build up the text draft. DC assisted in all wet-lab experiments. HJ provided the pre-enrichment material. AB, HJ, LG-R, and EB-E contributed to the project design and helped in drafting the manuscript. EB-E coordinated the project and gave financial support. DJ coordinated and conceived

the project and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.744075/full#supplementary-material

Supplementary Figure S1 | Relative proportion (%) of the most common lactic acid bacteria (LAB) found in silage processes. Metagenomic sequence taxonomic assignment was carried out by using RefSeq database.

Supplementary Figure S2 | Secreted endo-enzymatic activities (OD values) on **(A)** six chromogenic polysaccharide hydrogels (CPH) and **(B)** two insoluble biomass substrates (ICB) after T10 consortium growth on unmodified pH 6.2, pH 5, and pH 4.

Supplementary Figure S3 | Relative proportion (16S rRNA data) of taxa (class) after incubation of the consortium at T10 in a range of pH values. Error bars represent SDs from three biological replicates.

Supplementary Table S1 | Detailed features of metagenome assembled genomes (MAGs) reconstructed from T2, T10, and S consortia.

Supplementary Table S2 | Number of genes involved in lactic acid production found within Bin6_S, Bin002_S, Bin2_S, Bin4_T10, and Bin44_S.

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Effect of Different Regions and Ensiling Periods on Fermentation Quality and the Bacterial Community of Whole-Plant Maize Silage

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This study aimed to explore the changes in the microbial community on the silage material surface and during the ensiling process of whole-plant maize in different regions. Whole-plant maize silages were sampled in Ziyun, Guanling, and Weinning counties within warm and humid climate areas in southern China. Silages were sampled at 0, 2, 5, 10, 20, and 45 days during ensiling. The nutritional components, fermentation properties, and microbiomes were examined to evaluate the influence of sampling area and fermentation time on the quality of silage. The results showed that the pH values of all silages significantly decreased (<4.2 at ensiling day 2) during fermentation and all silages achieved satisfactory fermentation at 45 days. Butyric acid was not detected during ensiling, and the contents of acetic acid and ammonia nitrogen in the final silages were below 6 g/kg DM and 50 g/kg total nitrogen, respectively. *Weissella* was the dominant epiphytic bacteria of raw material in Ziyun and Weinning, while *Lactobacillus* was prevalent in Guanling. *Lactobacillus* dominated the ensiling process, and its abundance significantly increased with increasing fermentation time in the three groups. *Lactobacillus* was negatively correlated with pH of all silages ($p < 0.05$) and positively correlated with lactic acid, propionic acid and acetic acid ($p < 0.05$). Furthermore, the bacterial community was significantly correlated with environmental factors. Altitude had a highly positive correlation with the abundance of *Stenotrophomonas*, *Chryseobacterium,* and *Massilia* ($p < 0.01$), while precipitation was negatively correlated with these bacteria. The humidity and average temperature significantly influenced the *Lactobacillus* and *Weissella* abundances of fresh whole-plant maize. During the ensiling process, the silages from three regions had similar bacterial dynamic changes, and the *Lactobacillus* formed and maintained good fermentation characteristics in whole-plant maize silage.

Keywords: whole-plant maize, silage, fermentation quality, microbial community, environmental factor

INTRODUCTION

Ensiling is a common method for preserving fresh forage and contributes to an uninterrupted supply of forage feedstuff to ruminant animals (Pahlow et al., 2003). Lactic acid bacteria (LAB) are the most important beneficial bacteria in the process of ensiling fermentation (Zhu et al., 2010), which results in organic acids with water-soluble carbohydrate (WSC) as fermentation substrate under anaerobic conditions to reduce pH value for achieving the purpose of long-term preservation of silage (Keshri et al., 2018). It is well established that LAB regularly relate

to silage fermentation belong to the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus,* and *Weissella* (Pang et al., 2011; Ni et al., 2018; Yang et al., 2019). The quality of silage mainly depends on the composition and abundance of microbial communities during ensiling (Kung et al., 2018; Zi et al., 2021a). Whole-plant maize silage has a better absorption rate and higher nutritional value than other silages, and is the most common feed for ruminants worldwide (Khan et al., 2015; Zhang et al., 2019). Various silage microbial compositions have been found in different forages (Guo et al., 2018; Xu et al., 2019; Yuan et al., 2019). In addition, for maize ensiling, previous studies have reported that *Lactobacillus* was the dominant contributors during ensiling and subsequent exposure to air (Lin et al., 1992; Zhou et al., 2016; Hu et al., 2018). Generally, the composition of microorganisms changes greatly before and after ensiling (Sepehri and Sarrafzadeh, 2019). Therefore, it is of great significance to further study the microbial diversity changes in silage for understanding the whole fermentation process and finding the root cause of fermentation quality changes.

As previously mentioned, uncontrollable climatic conditions affect silage fermentation, microorganisms and aerobic stability (Bernardes et al., 2018; Guan et al., 2018). Ensiling at high temperatures reduces both the lactic acid (LA) concentration and aerobic stability (Ashbell et al., 2002). Moreover, it has been reported that temperature affects the bacterial diversity and fermentation quality of silage (Wang et al., 2019; Li et al., 2021). Epiphytic bacterial communities in fresh forage depends mainly on geographical location, climate, and cutting (Guo et al., 2018; Yang et al., 2019). Cai et al. (1999) studied the microorganisms attached to the surface of corn, sorghum, alfalfa and Italian ryegrass collected from the same place and found that there were very few *Pediococcus* attached to the surface of sorghum and ryegrass and few *Lactobacillus* attached to the surface of alfalfa. The community of epiphytic bacteria in corn material has been shown to be affected by rainfall and humidity, and the microbial community during ensiling has been shown to be affected by temperature (Guan et al., 2018).

The composition of the epiphytic community is an important factor of silage quality and changes in the microbial community during fermentation (Gharechahi et al., 2017). Nevertheless, the effect of the environment on the epiphytic community of whole-plant maize silage has rarely been reported. We hypothesized that the succession characteristics of whole-plant maize silage are different in different areas. Therefore, this study aimed to evaluate the correlation between bacterial communities and sampling area environmental factors (altitude, precipitation, temperature, and humidity) and then to examine the dynamic changes in microbial community diversity and fermentation quality during the ensiling of whole-plant maize.

MATERIALS AND METHODS

Study Sites and Sample Collection

Whole-plant maize was planted in Ziyun County (Z) (106°10′E, 25°37′N, altitude 840 m), Guanling County (G) (105°24′E, 25°57′N, altitude 1350 m), and Weining County (W) (104°16′E, 26°55′N, altitude 2230 m). The monthly changes of temperature, precipitation and humidity in the three regions in 2019 are shown in **Figure 1**. The variety of maize was Qingfeng No. 4. The samples were planted on February 25, 2019; March 28, 2019; and April 2, 2019 in Ziyun, Guanling, and Weining, respectively. Samples were collected at the dough stage according to local tradition on July 31, 2019; September 12, 2019; and September 18, 2019. Instantly, the materials were collected and transported in ice boxes and stored at –80°C before use. Another part of the collected materials was chopped to approximately 2 cm by a hand paper cutter. After mixing thoroughly, the material was vacuum-packed into plastic bags. Each bag contained approximately 500 g of fresh matter. A total of 54 bags (three treatments × six ensiling durations × three replicates) were prepared and stored at normal temperature (25–30°C), and the fermentation time of all samples was approximately 45 days. Samples were used to determine the chemical composition, fermentation quality and microbial community on days 0, 2, 5, 10, 20, and 45.

Chemical and Fermentation Profile Analyses

Specimens were determined by drying the samples in a forced air oven at 65°C for 72 h and passed through a 1.0 mm sieve before the chemical assay. The contents of dry matter (DM) and crude protein (CP) were measured according to previously published study (AOAC, 2000). The WSC content was determined by colorimetric after-reaction with anthrone reagent (Turula et al., 2010). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed using a previously established method (Van Soest et al., 1991). To measure fermentation quality, 20 g samples were suspended in 180 mL of distilled water overnight at 4°C and filtered through four layers of cheesecloth. Then, the filtrates were used to determine the pH value and ammonia-N (NH_3-N) and organic acid contents. The pH, lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA), ammonia-N (AN), and total nitrogen (TN) concentrations were measured as previously established (Li M. et al., 2019).

Bacterial Community Analysis

The DNA extraction was operated using the HiPure Soil DNA extraction kit (Magen, Guangzhou, China) according to the manufacturer's instructions. The 16S rDNA V5-V7 region of the ribosomal RNA gene were amplified by PCR (94°C for 2 min, followed by 30 cycles at 98°C for 10 s, 62°C for 30 s, and 68°C for 30 s and a final extension at 68°C for 5 min) with primers 799F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC) (Logue et al., 2016). Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and quantified using ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, United States). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina platform according to the standard protocols.

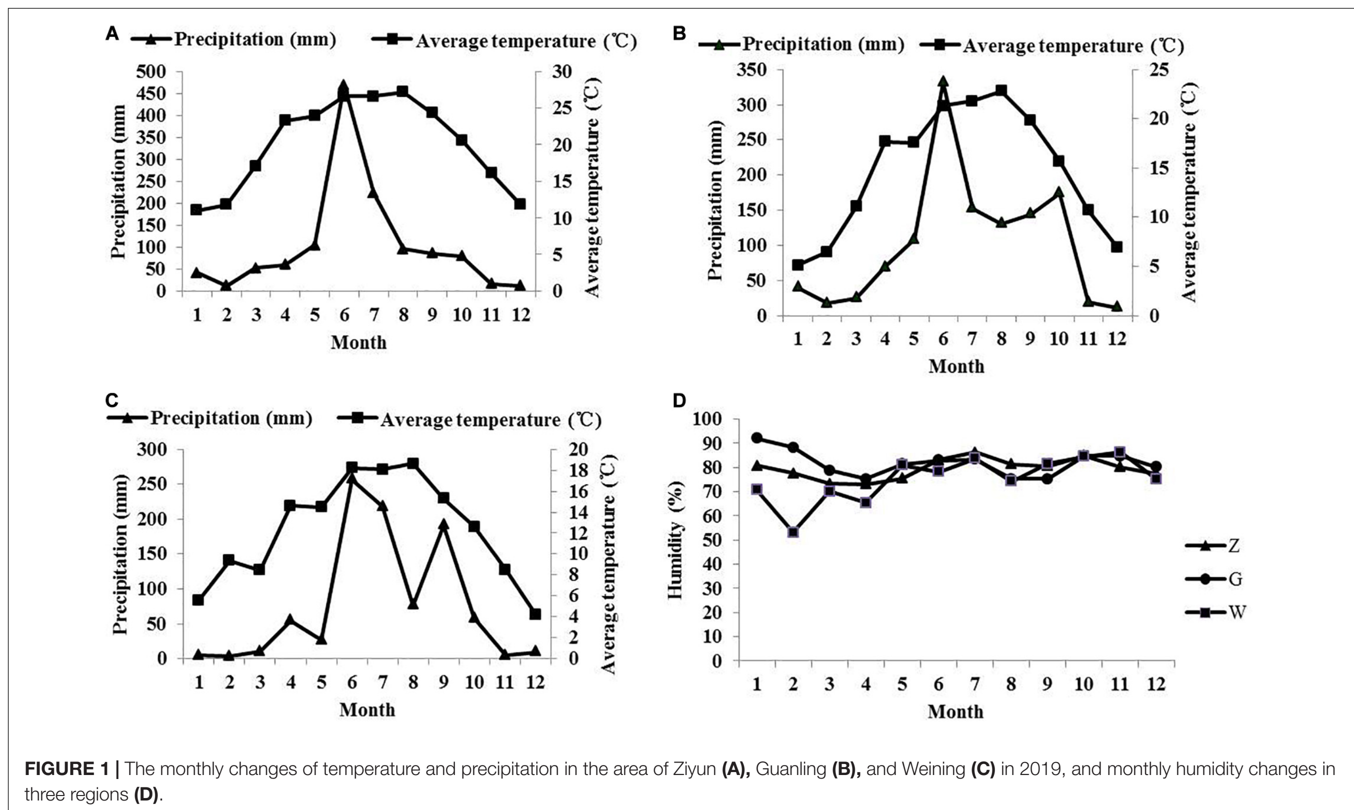

FIGURE 1 | The monthly changes of temperature and precipitation in the area of Ziyun **(A),** Guanling **(B),** and Weining **(C)** in 2019, and monthly humidity changes in three regions **(D)**.

Tag assembly was carried out using filtered reads according to the following principles: the overlap between paired-end reads should be more than 10-bp and have less than a 2% mismatch. The unique tags were obtained by removal of redundant tags using MOTHUR software (Schloss et al., 2009). The effective tags were clustered into operational taxonomic units (OTUs) of ≥97% similarity using UPARSE (version 9.2.64) pipeline. Diversity metrics were determined using the core-diversity plug-in within QIIME (Callahan et al., 2016). The microbial diversity within an individual sample was assessed using the following alpha diversity indices: the Chao1 richness estimator and Shannon diversity index. Beta diversity was analyzed to assess the structural variation of microbiota across specimens, and then principal component analysis (PCA) was conducted (Vázquez-Baeza et al., 2013). The relative abundances of different bacterial communities at the phylum and genus levels were also analyzed. Pearson correlation coefficient between environmental factors and species was calculated in R project psych package (version 1.8.4). The heat map function of the R software and genus information for the Whole-plant silages were used to generate a heat map. Environmental factors during corn growth and fermentation quality after silage were selected for Spearman correlation analysis with bacterial community. The data were analyzed using the free online platform of Omicshare tools.

Statistical Analyses

The statistical analyses of two-way ANOVA were performed using SPSS 20.0. Duncan's HSD test was employed to determine the differences in the treatment means, where significant differences were declared at $P < 0.05$, and the data are expressed as the mean and the standard error of the mean (SEM).

RESULTS

Chemical Analysis of Whole-Plant Maize Ensiling

The changes in nutritional components during different regions of ensiling are shown in **Table 1**. The DM contents of all maize raw materials ranged from 268.6 to 303.3 g/kg, while the highest DM content was 303.3 g/kg in the W group ($P < 0.05$), and the lowest DM content was 268.6 g/kg in the G group ($P < 0.05$). The content of DM in each group decreased with increasing fermentation time and was in the order of W > Z > G samples at any stage of fermentation. The content of CP in the Z and G groups was lower than that in the W group on days 5 and 10 ($P < 0.05$), while that in the Z group was lower than that in the G and W groups on day 45 ($P < 0.05$). The WSC content of each group decreased significantly throughout the fermentation process. The contents of NDF and ADF in the Z group were significantly higher than those in the other groups at any stage of fermentation, and they showed a similar decreasing trend with the extension of fermentation time. Moreover, the contents of NDF and ADF in the G group were lower than those in the other groups on day 45 ($P < 0.05$). The ensiling time (D) and treatment (T) significantly affected the chemical composition ($P < 0.001$).

TABLE 1 | Chemical composition of whole-plant maize silage in different regions.

Item	Treatment	Ensiling days						SEM	*P*-value		
		0	2	5	10	20	45		D	T	D × T
Dry matter (g/kg FW)	Z	274.1Ba	268.6Bb	262.0Bc	254.9Bd	254.4Bd	250.9Be	0.36	***	***	***
	G	268.6Ca	261.7Cb	254.5Cc	252.6Cd	250.7Ce	248.6Cf				
	W	303.3Aa	293.6Ab	292.1Ac	274.8Ad	264.7Ae	260.6Af				
Water-soluble carbohydrate (g/kg DM)	Z	86.9Aa	66.0Ab	59.0Ac	42.6Ad	32.9Ae	25.2Af	0.42	***	***	***
	G	78.9Ba	58.4Bb	48.7Bc	40.1Bd	33.3Ae	22.6Bf				
	W	72.0Ca	51.4Cb	39.7Cc	35.4Cd	31.5Be	21.3Bf				
Crude protein (g/kg DM)	Z	74.3Cb	71.3Bb	74.0Cb	83.1Ba	72.9Cb	72.5Cb	0.74	***	***	***
	G	84.5Aa	81.8Ab	78.5Bc	76.6Cd	79.1Bc	78.7Ac				
	W	78.4Bc	73.7Bd	83.0Ab	90.1Aa	89.0Aa	74.2Bd				
Starch (g/kg DM)	Z	267.9Ca	262.0Ab	259.4Bc	258.7Bc	255.6Bd	240.8Be	1.17	***	***	***
	G	275.0Ba	252.0Bbc	252.9Cb	250.6Ccd	248.7Cd	244.1Be				
	W	296.2Aa	260.8Ac	272.4Ab	275.3Ab	276.1Ab	275.1Ab				
Neutral detergent fiber (g/kg DM)	Z	527.1Aa	525.7Aa	522.4Aa	515.8Ab	511.0Ab	475.0Ac	1.73	***	***	***
	G	504.1Ba	496.5Bb	495.0Bb	488.5Bc	476.0Bd	456.2Be				
	W	477.3Ca	461.5Cb	457.8Cb	444.0Cc	439.7Cd	437.1Cd				
Acid detergent fiber (g/kg DM)	Z	257.9Aa	255.4Ab	252.9Ac	250.2Ad	248.6Ae	246.9Af	0.64	***	***	***
	G	250.7Ba	244.3Bb	236.4Bc	226.6Cd	222.4Ce	207.3Cf				
	W	244.3Ca	237.4Cb	232.4Cc	229.5Bd	227.4Be	225.4Bf				

*Z, Ziyun; G, Guanling; W, Weining; FM, fresh matter; DM, dry matter; ND, not detected; "–", default. D, ensiling days; T, treatment; D × T, interaction of ensiling days and treatment; SEM, standard error of means; Means with different letters in the same row (a–f) or column (A–C) differ ($P < 0.05$), ***$P < 0.001$.*

The results also showed a significant interaction between D and T for the contents of DM, WSC, NDF, and ADF ($P < 0.001$).

The Fermentation Property of Whole-Plant Maize During Ensiling

Table 2 illustrates the fermentation quality of whole-plant maize silage in different regions. The pH value of each group decreased significantly on the second day of fermentation ($P < 0.05$), all of which were below 4.2. Moreover, the highest and lowest pH appeared in the G group and the W group on day 45 ($P < 0.05$), respectively. The LA content of each group increased significantly during fermentation, and the highest LA content was found in the W group on day 45 ($P < 0.05$). The contents of AA and PA in each group gradually increased with prolonged ensiling, and the contents in the Z group were lower than those in the other groups on day 45 ($P < 0.05$). The contents of BA were not detected in all silages. The contents of AN/TN in all groups were dramatically increased ($P < 0.05$) during fermentation, within lower than 5%. Moreover, pH, AA, LA, PA, and AN/TN were interactively influenced by treatment and ensiling days ($p < 0.001$).

Microbial Diversity of Whole-Plant Maize During Ensiling

The bacterial alpha diversities of the silages were evaluated by the Chao1 and Shannon indexes (**Figures 2A,B**). The bacterial diversity increased with the extension of fermentation time in the Z group. However, the bacterial diversity of silages in the G group and the W group was richer after 2 days of fermentation.

The relative abundances of bacterial communities at the phylum and genus levels were presented in **Figures 3**, **4**. As seen in **Figure 3**, Proteobacteria and Firmicutes were the top two phyla during the process of ensiling in the three regions. *Lactobacillus*, *Weissella,* and *Acetobacter* were the most dominant genera in all maize silages. **Figure 4A** shows the abundance of Proteobacteria was the most abundant phylum in the G group, exceeding 95% in raw materials. The dominant bacteria on the surface of maize in the Z and W groups were Firmicutes, with relative abundances of 90.55 and 71.70%, respectively, and Proteobacteria had relative abundances of 9.22 and 27.04%, respectively. At the genus level, *Weissella* and *Lactobacillus* were found in large amounts in the Z and W groups, while no obvious dominant bacteria were found in the G group of raw materials.

All the groups were dominated by Firmicutes and Proteobacteria during ensiling at the phylum level. The Proteobacteria abundance decreased while that of Firmicutes increased rapidly and became the dominant phylum with increasing fermentation time. At the genus level, the most dominant bacterial genera were *Lactobacillus* and *Weissella* in the samples during the ensiling process. After ensiling, *Lactobacillus* was the dominant microbial genus, and its relative abundance exceeded 70%. The *Lactobacillus* abundance significantly increased with the extension of fermentation time, while those of *Weissella* and *Acinetobacter* significantly decreased

TABLE 2 | Fermentation quality of whole-plant maize silage in different regions.

Item	Treatment	Ensiling days						SEM	P-value		
		0	2	5	10	20	45		D	T	D × T
pH	Z	5.60Aa	3.76Cc	3.72Bc	3.67Bd	3.62Ce	3.80Bb	0.015	***	***	***
	G	5.59Aa	4.03Ab	3.87Ac	3.83Ac	3.78Ad	3.88Ac				
	W	5.56Aa	3.91Bb	3.73Bc	3.67Bd	3.66Bd	3.52Ce				
Lactic acid (g/kg DM)	Z	ND	31.5Ad	33.3Ac	34.4Ab	36.0Aa	29.2Be	0.179	***	***	***
	G	ND	22.5Ce	26.0Bc	27.7Bb	30.2Ca	23.5Cd				
	W	ND	24.9Bd	33.2Ac	34.3Ab	34.8Bb	36.1Aa				
Acetic acid (g/kg DM)	Z	ND	1.6Ae	2.4Ad	2.7Ac	3.4Ab	4.5Ca	0.067	***	***	***
	G	ND	1.3Be	1.9Bd	2.3Bc	2.9Bb	5.5Aa				
	W	ND	1.7Ad	2.4Ac	2.6Ac	3.7Ab	4.9Ba				
Propionic acid (g/kg DM)	Z	ND	0.8Cd	1.1Bc	1.4Bb	1.6Bb	1.9Ba	0.058	***	***	***
	G	ND	1.1Bd	1.9Ac	2.1Ac	2.3Ab	2.7Aa				
	W	ND	1.4Ae	1.7Ad	1.9Ac	2.2Ab	2.5Aa				
Butyric acid (g/kg DM)	Z	ND	ND	ND	ND	ND	ND	—	—	—	—
	G	ND	ND	ND	ND	ND	ND				
	W	ND	ND	ND	ND	ND	ND				
Ammonia-N (g/kg total N)	Z	15.9Be	19.5Bd	23.0Bc	23.7Bc	27.0Bb	34.4Ba	0.393	***	***	***
	G	15.5Bf	22.5Ae	25.1Ad	30.9Ac	34.8Ab	41.7Aa				
	W	20.1Ae	23.0Ad	23.9Bcd	24.8Bc	26.5Bb	31.9Ca				

*Z, Ziyun; G, Guanling; W, Weining; FM, fresh matter; DM, dry matter; ND, not detected; "–", default. D, ensiling days; T, treatment; D × T, interaction of ensiling days and treatment; SEM, standard error of means; Means with different letters in the same row (a–f) or column (A–C) differ ($P < 0.05$), ***$P < 0.001$.*

or disappeared in the subsequent period. The relative abundance of *Acetobacter* in each group decreased with the process of ensiling. By 45 days, the relative abundance of *Acetobacter* in each group decreased to below 1%. In addition, *Pantoea* and *Stenotrophomonas* existed in the whole corn silage period of all three regions.

Correlation Analysis Between Bacterial Communities and Environmental Factors

The association analysis between bacterial abundance and environmental factors is shown in **Figure 5**. Negative correlations were observed between the average temperature and the relative abundance of the *Lactobacillus* (–0.73), *Sphingomonas* (–0.67), *Stenotrophomonas* (–0.74), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (–0.92), *Chryseobacterium* (–0.75), and *Massilia* (–0.71). Altitude was associated with increased abundance of *Rahnella, Stenotrophomonas*, *Chryseobacterium*, *Massilia* ($p < 0.05$), *Serratia*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* ($p < 0.01$). The level of precipitation was associated with decreased abundance of *Rahnella*, *Serratia* ($p < 0.01$) and *Stenotrophomonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Chryseobacterium* and *Massilia* ($p < 0.05$). Humidity was associated with increased abundance of *Weissella*, *Lactobacillus*, *Stenotrophomonas*, *Chryseobacterium*, *Massilia* ($p < 0.05$), and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* ($p < 0.001$), while it was associated with decreased abundance of *Escherichia–Shigella* ($p < 0.05$).

Correlation Analysis of Bacterial Community With Fermentation Products

The association analysis between bacterial abundance and fermentation indexes is shown in **Figure 6**. Specifically, the pH value was associated with decreased abundance of *Lactobacillus* ($p < 0.01$). The LA concentration was associated with increased abundance of *Lactobacillus* ($p < 0.05$), while it was negatively correlated with that of *Pseudomonas* and *Massilia* ($p < 0.05$). The contents of AA and PA were associated with increased abundance of *Lactobacillus* ($p < 0.001$), while AA concentration was negatively correlated with the abundance of *Acetobacter* and *Pseudomonas* ($p < 0.01$) and *Pantonea* ($p < 0.05$), PA concentration was associated with decreased abundance of *Acetobacter*, *Pseudomonas* and *Lysinibacillus* ($p < 0.01$) and *Weissella*, *Gluconobacter*, and *Oceanobacillus* ($p < 0.05$). Finally, the positive correlations have been observed between AN/TN concentrations and *Lactobacillus* ($p < 0.01$) and *Herbaspirillum* ($p < 0.05$), while AN/TN concentrations was associated with decreased abundance of *Acetobacter*, *Rahnella*, *Pseudomonas*, *Lysinibacillus,* and *Pantoea* ($p < 0.05$).

DISCUSSION

Nutritional Characteristics of Whole-Plant Maize During Ensiling

The expected DM content for good silage is 30∼35% (Guyader et al., 2018). The DM content in each group decreased with the

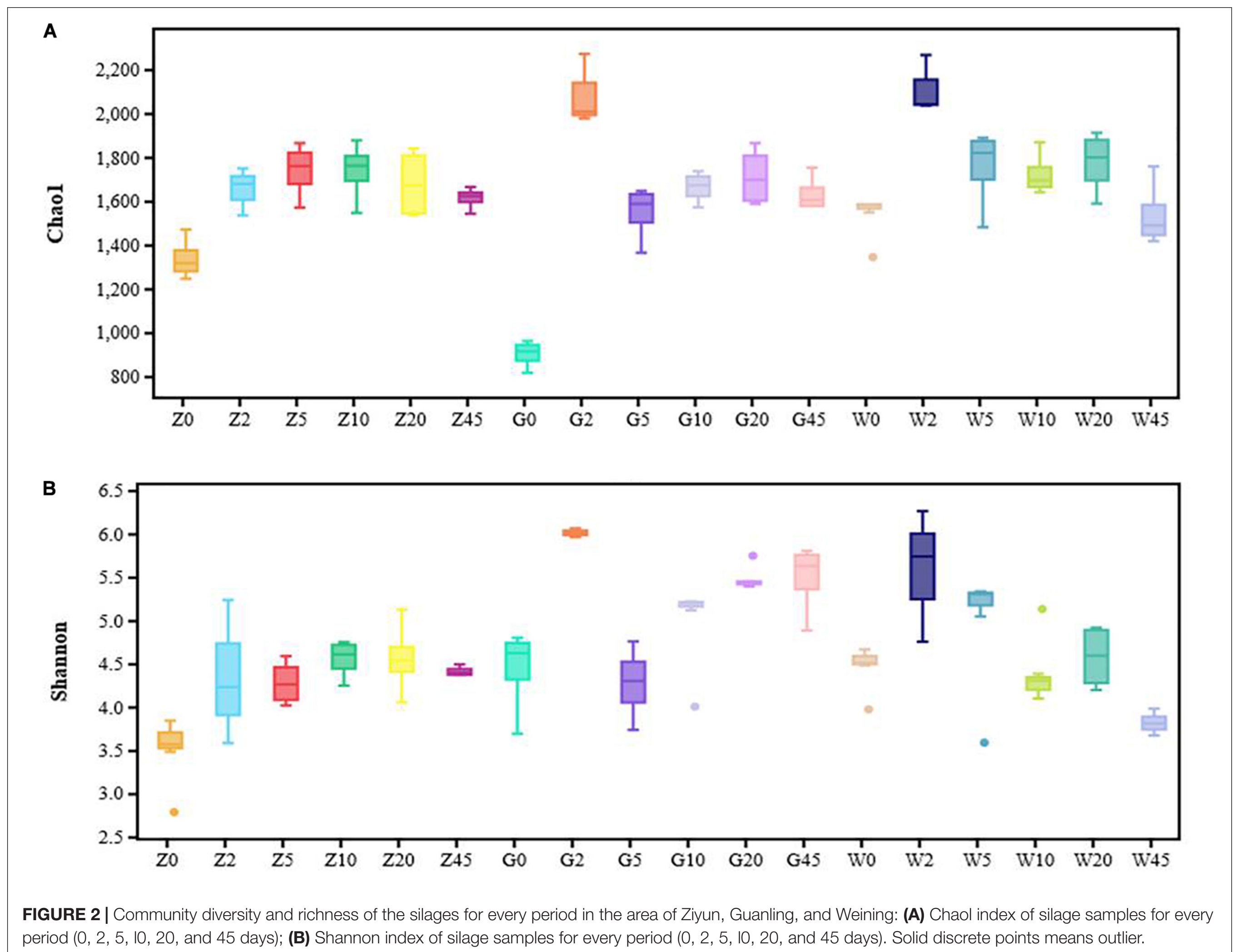

FIGURE 2 | Community diversity and richness of the silages for every period in the area of Ziyun, Guanling, and Weining: **(A)** Chaol index of silage samples for every period (0, 2, 5, l0, 20, and 45 days); **(B)** Shannon index of silage samples for every period (0, 2, 5, l0, 20, and 45 days). Solid discrete points means outlier.

extension of fermentation time, which was mainly due to the WSC being consumed by LAB and other microorganisms for fermentation (Hu et al., 2009). The different CP contents of raw materials may be related to cultivation and fertilization (Miao et al., 2006). CP is one of the main nutritional components of silage, and the degradation of protein will affect the nutritional value of pastures. The lost nitrogen can only meet the nutritional needs of livestock by supplementing protein feed in the diet, thus increasing the breeding cost (Bilal, 2009). In this study, the CP content of the Z and G groups increased on the 10th and 20th days, respectively, while that of the W group increased on the 5th and 10th days of fermentation, which may be due to the decrease in DM content (Zhang et al., 2018). In addition, when the pH was low, some bacteria composed of protein could not grow and reproduce in the fermentation process and became a part of the feed, which also increased the content of CP (Bilal, 2009). On the other hand, the loss in CP was relatively small due to good fermentation.

The WSC content decreased significantly throughout the fermentation process, and the loss rate of each group reached 70%. As the substrate of silage fermentation, the WSC content will be decomposed by LAB to produce LA (Moselhy et al., 2015). The content of ADF and NDF affects the chewing time of ruminants and indirectly affects feed digestibility in domestic animals (Jang et al., 2017). In the present study, the contents of NDF and ADF in each group decreased with increasing fermentation time, which indicated that silage fermentation had a certain degradation effect on the fiber components of whole-plant maize, thus improving the digestibility of silage.

The Fermentation Property of Whole-Plant Maize During Ensiling

The pH value of silage is an important index to evaluate the success of silage, and well-fermented silage should have a pH of 3.8∼4.2 (Kung et al., 2018). The low pH value ensures that harmful bacteria are inhibited and finally contributes to the good fermentation of whole-plant maize (Keshri et al., 2018). The pH was less than 4.0 and reached the lowest point at 20 days in all the silages except for that in the W group, which agreed with the results of a previous study (Sun et al., 2021).

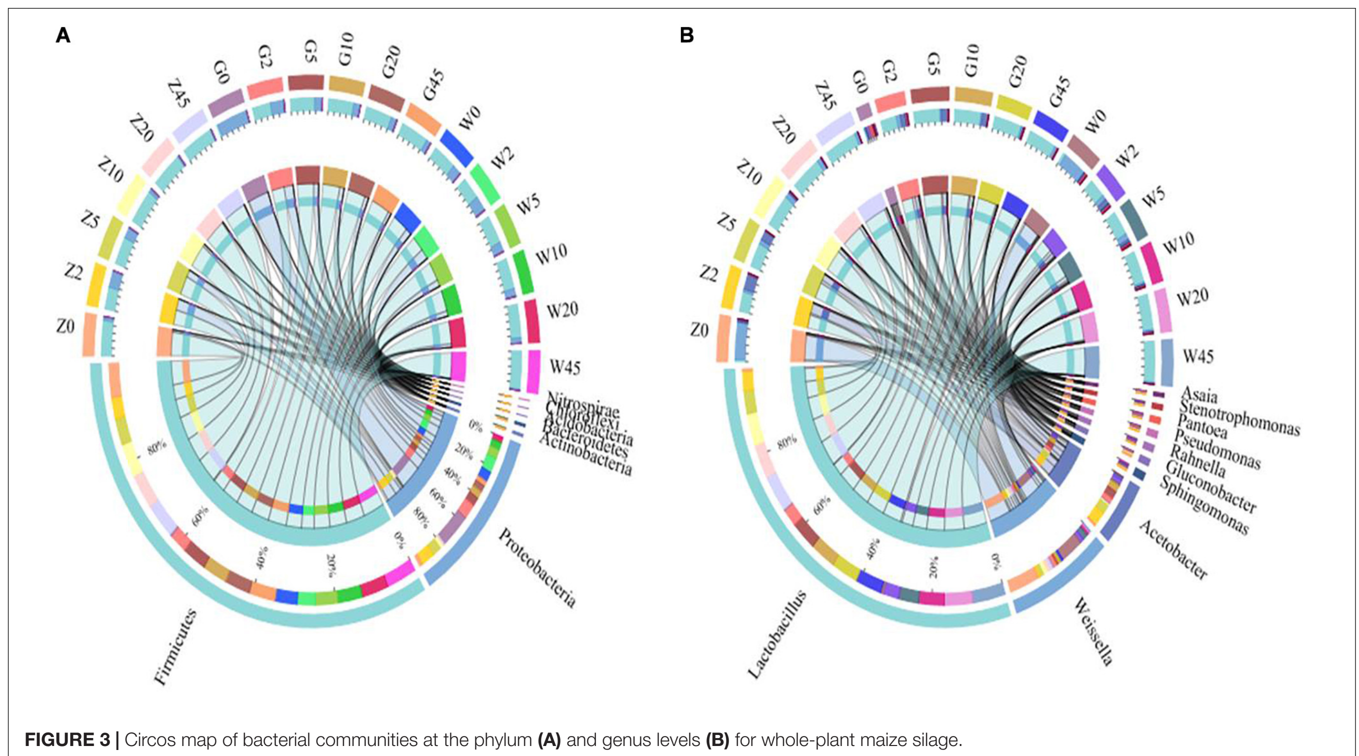

FIGURE 3 | Circos map of bacterial communities at the phylum **(A)** and genus levels **(B)** for whole-plant maize silage.

FIGURE 4 | The relative abundance of bacteria community proportions at the phylum **(A)** and genus **(B)** level across the treatments (as a percentage of the total sequence).

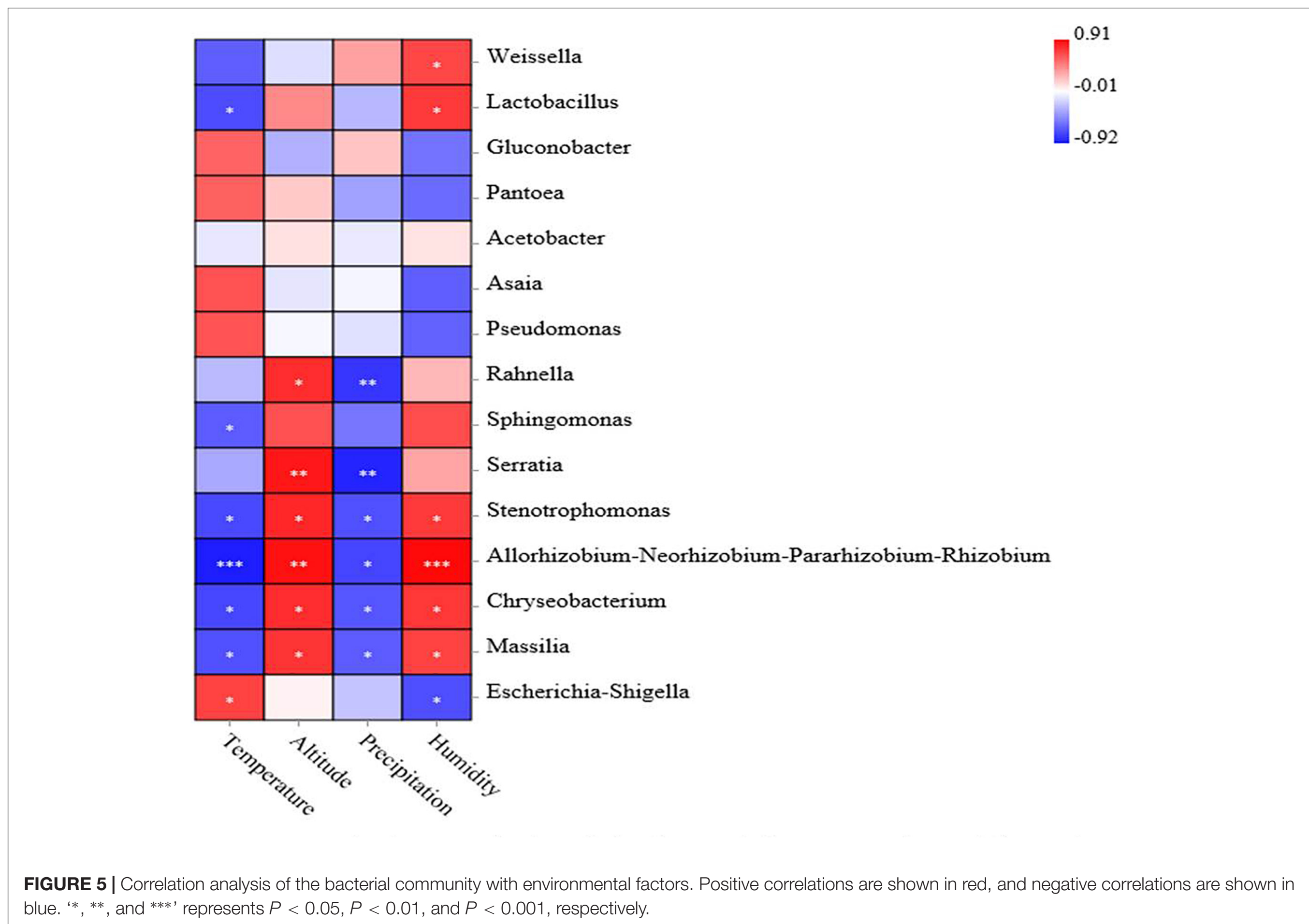

FIGURE 5 | Correlation analysis of the bacterial community with environmental factors. Positive correlations are shown in red, and negative correlations are shown in blue. '*, **, and ***' represents $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

According to the fermentation mode, LA fermentation can be divided into homofermentative and heterofermentative types (Guo et al., 2018). Homofermentation mainly produces LA, whereas heterofermentation also produces AA, ethanol and CO_2 in addition to LA (Pahlow et al., 2003). In this study, LAB grew rapidly and produced enough LA in each group, which inhibited the growth and reproduction of harmful microorganisms. With increasing LA content, homofermentative LAB are inhibited, while heterofermentative LAB begin to dominate due to the stronger tolerance to AA and pH value, and fermentation gradually changes from homofermentative LAB fermentation to heterofermentative LAB fermentation (Shao et al., 2002). The AA content of each group gradually increased with increasing fermentation time. In addition, when the silage is fermented to a certain extent and the pH is low, the fermentation of LAB will also be inhibited. At the same time, some anaerobic microorganisms that may exist in silage begin to decompose LA and produce other organic acids, such as AA and PA, which leads to a decrease in LA content (Shao et al., 2002). In this study, with the extension of fermentation time, the LA content decreased and the AA and PA contents increased gradually in the G group, which indicated that the type of fermentation was heterofermentative. The contents of LA and AA were relatively high, and the ammonia nitrogen content was within a limited range. These indexes showed the excellent fermentation quality of all the silages. BA is a product produced by decomposing protein, glucose and LA by spoilage bacteria and BA bacteria, respectively (Addah et al., 2012). It has been reported that BA content > 5 g/kg DM affects the palatability of feed and reduces the feed intake by livestock (Muck, 2010). During the whole fermentation process, BA was not detected in any group.

During the ensiling process, the ammonia nitrogen is mainly produced by the degradation of protein by plant enzymes and the utilization of protein and amino acids by microbial decomposition (Kung et al., 2018). In general, the ammonia-N content is recommended to be less than 5% for maize silage (Zhang et al., 2016). The high ammonia-N content indicates that proteolysis occurred to a deep extent during ensiling (Mu et al., 2021). In the present study, the content of AN/TN in each group was lower than 5%, indicating that harmful bacteria were effectively inhibited.

Microbial Diversity of Whole-Plant Maize During Ensiling

The microbe numbers and species composition are varied with different silage materials or ensiling processes (Khota et al., 2016). Various epiphytic bacterial communities in raw

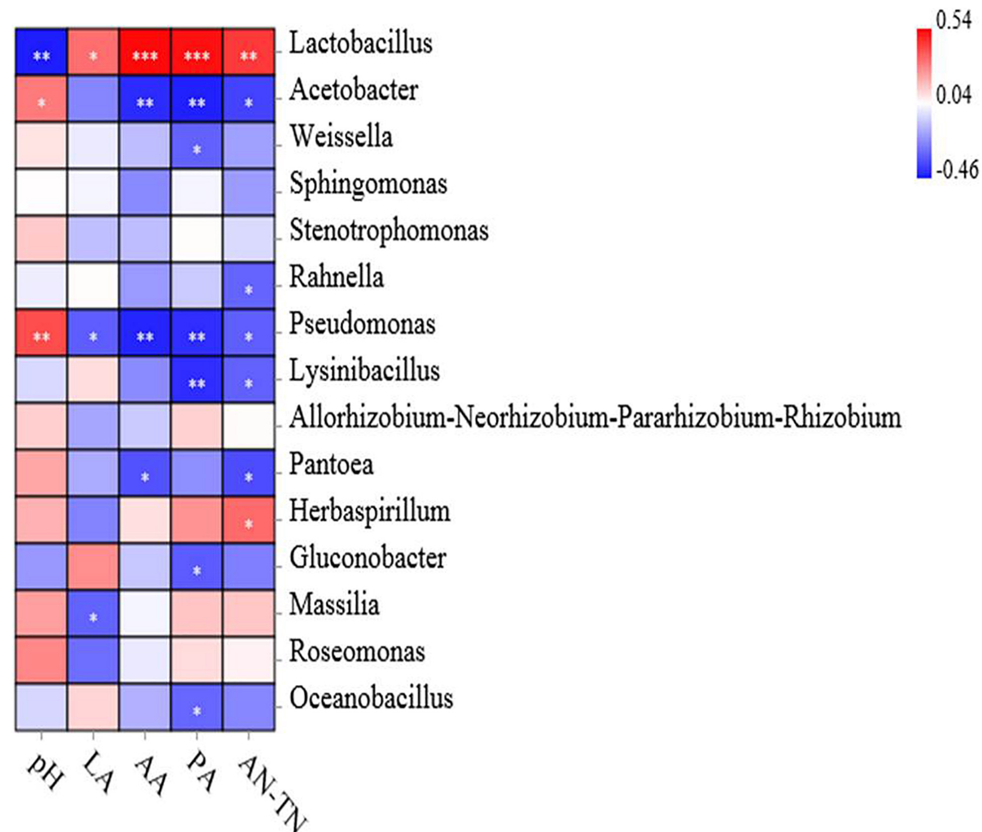

FIGURE 6 | Relationships of fermentation characteristics with silage bacterial community at the genus level. Positive correlations are shown in red, and negative correlations are shown in blue. '*, **, and ***' represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

materials might originate from unique growth environments (McGarvey et al., 2013). Gharechahi et al. (2017) reported a special bacterial community in whole-plant corn from different geographical locations. In this study, the differences at the species level in the epiphytic microorganisms of the Whole-plant fresh samples from the three areas were observed. The sampling locations in three areas belong to a subtropical monsoon climate and are close to each other. The phylum-level bacteria mainly attached to the surface of silage maize in the three regions were Firmicutes and Proteobacteria. Additionally, the main LAB genus in fresh forages was *Weissella*. This indicated that *Weissella* might play a major role in the early stage of fermentation (Sun et al., 2021). This genus is a selective anaerobic bacterium, which converts water-soluble sugars into LA and AA in the early stage of silage fermentation (Cai et al., 1998). In the current study, *Weissella* with low abundance was detected in silages during the ensiling process (**Figure 4**), which might lead to relatively high AA content in all silages (**Table 2**).

The nature of silage is due to complex microbial succession (Yang et al., 2019), in which bacteria play a key role (Li P. et al., 2019). In this study, the abundance of Firmicutes increased rapidly and became the dominant phylum during ensiling. This observation might be explained by the fact that the growth of Firmicutes is related to the low pH and anaerobic conditions formed during ensiling (Wang et al., 2019; Zhang et al., 2019). Although *Lactobacillus* was usually not the dominant bacteria in fresh corn of the Z group, it began to dominate at 2 days of ensiling. This observation suggested that the LAB count in the raw materials is enough to ensure the fermentation quality of the final silage. *Lactobacillus*, *Lactococcus, Weissella,* and *Enterococcus* are common LA-producing bacteria in silage, and their abundance changes are closely related to the quality of silage (Ni et al., 2018). Generally, LA-producing cocci are the dominant LAB. These initiate LA fermentation during the early stages of ensiling, whereby *Lactobacillus* will grow rapidly into the dominant bacteria (Liu et al., 2019). This result suggested that *Lactobacillus* was an important genus for silage fermentation during ensiling. Many studies have shown the dominance of *Lactobacillus* in ensiled corn silages (Li and Nishino, 2011; Ogunade et al., 2017) due to its desirable functions during ensiling (Li et al., 2017; Liu et al., 2019). The study showed that *Lactobacillus* and *Weissella* were the only LAB with high abundance in the whole-plant maize silage process in different regions, which was consistent with the research of Ogunade et al. (2017), and the reason for the difference with the research of

Tohno et al. (2013) and Ni et al. (2017b) may be related to the research materials and locations.

Gluconobacter and *Acetobacter* were found in minor abundance during the early stages of ensilage. *Acetobacter* is a kind of AA-producing and nitrogen-fixing bacteria (Kumiko et al., 2001) and may lead to the decline in pH at the early stage of the ensiling process. Other bacteria, such as *Pantoea*, *Rahnella,* and *Sphingomonas*, were evenly distributed during the ensiling process in lower amounts are consistent with previous studies (Gharechahi et al., 2017; Ni et al., 2017a), and some of them are undesirable in silage (McDonald et al., 1991). Muck (2010) reported that *Bacillus*, *Paenibacillus*, *Enterobacter*, *Enterococcus,* and *Clostridium* are the main microorganisms that decompose proteins into ammonia nitrogen and cause protein loss. None of these bacteria were found in the top 10 bacteria regarding relative abundance, which was consistent with the previous results of low AN/TN contents in the present study.

Correlation Analysis Between Bacterial Communities and Environmental Factors

There are many factors (moisture, WSC, regional factors) that affect the microbiome and influence the fermentation quality of silage (McEniry et al., 2010; Bernardes et al., 2018). Usually, regional factors include temperature, humidity, precipitation, longitude, latitude, and altitude. Previous reports showed that high temperatures and rainfall had detrimental effects on the fermentation process and silage quality (Kim and Adesogan, 2006). In addition, high temperature affected the transformation of microorganisms in corn silage from a homofermentative to a heterofermentative LAB community, which had been previously reported (Guan et al., 2020). Guan et al. (2018) reported that *Weissella*, *Pseudomonas*, and *Lactobacillus* were the main epiphytic bacteria of corn silage in high-temperature and high-humidity areas. Moreover, Zi et al. (2021b) reported that temperature, humidity, and precipitation affected the fermentation quality of silage through the changes in microbiomes. Epiphytic bacterial communities are highly related to climate (Bernardes et al., 2018). In this study, altitude was positively correlated with the abundance of *Stenotrophomonas*, *Chryseobacterium,* and *Massilia*, while precipitation was negatively correlated with these bacteria. This indicates that precipitation was the factor affecting the epiphytic bacteria of the silage material. The *Lactobacillus* abundance in fresh maize did not change regularly with increasing altitude, and the reason for the difference may be related to the temperature and precipitation in the three regions. The correlation analysis (**Figure 5**) showed that the relative abundance of *Lactobacillus* was negatively correlated with temperature. This also explains why the temperature of the G group was the highest (21.35°C), while the relative abundance of attached *Lactobacillus* was the lowest (2.75%). The temperature of the W group was the lowest (17.32°C), while the relative abundance of attached *Lactobacillus* was the highest (63.77%). Altitude and precipitation influence some microorganisms in silage, but they do not relate to the main bacteria in silage, such as *Lactobacillus* and *Weissella*. This shows that when silage enters a completely anaerobic environment, the influence of climate factors on the main microorganisms will become smaller. Cai et al. (1999) reported that a certain amount of LAB in forage will have a great impact on silage fermentation. In this study, *Lactobacillus* and *Weissella* were correlated with temperature and humidity, and the most dominant bacterial genera were *Lactobacillus* and *Weissella* in the samples during the ensiling process. This result indicated that the main factors affecting the microbial diversity of silages were humidity and average temperature. Environmental factors affect the community of epiphytic bacteria on raw materials, which have a greater impact on the initial stage of silage fermentation (Zi et al., 2021b). We speculate that the influence of environmental factors on the microbial community will decrease during the silage fermentation process, thus reducing its influence on silage quality.

Correlation Analysis of Bacterial Community With Fermentation Products

Microorganisms affect silage quality through a series of metabolites. For example, *Lactobacillus* species mainly affect LA production (Guan et al., 2018). In the present study, The *Lactobacillus* was positively correlated with the LA content, which dominated the bacterial community in the fermentation process and had a negative correlation with pH in all silages. This result was consistent with the report of Sun et al. (2021). *Pseudomonas* is an undesirable bacterium that can survive in an anaerobic environment, and its production of biogenic amines leads to the decrease in protein content (Roberson and Firestone, 1992; Dunière et al., 2013). The AN/TN content was negatively correlated with the abundance of *Pseudomonas*, indicating that the existence of *Pseudomonas* may contribute to the preservation of protein (Ogunade et al., 2018). Other studies have confirmed these findings, indicating that the fermentation characteristics are highly correlated with the microflora of silage and affect the overall fermentation quality (Ren et al., 2019; Yang et al., 2019).

CONCLUSION

All of the whole-plant maize silages had satisfactory fermentation quality. The bacterial community of fresh raw material was mainly composed of *Weissella* and *Proteobacteria*. Although the bacterial community varied during ensiling, *Lactobacillus* dominated the ensiling process. *Lactobacillus* had a negative correlation with pH in all silages and grew well under low pH conditions, produced LA during ensiling, and effectively influenced fermentation quality. Altitude and precipitation influenced some specific microorganisms in silage, but they did not affect the main bacteria in silages. The humidity and average temperature significantly influenced the abundances of *Lactobacillus* and *Weissella* of fresh whole-plant maize and had a greater impact on the whole fermentation process.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JH and CC contributed to conception and design of the study. LL, SD, and CW performed the statistical analysis. YH wrote the manuscript and interpretation of data. All the authors read and contributed to the manuscript.

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Effects of Phenyllactic Acid, Lactic Acid Bacteria, and Their Mixture on Fermentation Characteristics and Microbial Community Composition of Timothy Silage

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This study investigated the effects of phenyllactic acid (PL), lactic acid bacteria (LAB), and their mixture on fermentation characteristics and microbial community composition of timothy silage. Timothy silages were treated without (CK) or with PL [10 mg/kg fresh matter (FM) basis], LAB inoculant (IN; a mixture of *Lactobacillus plantarum* and *L.buchneri*, 10^5 cfu/g FM), and their mixture (PI) and stored at ambient temperature (5℃∼15℃) in a dark room for 60 days. Compared with CK, all treated silages showed lower ($P < 0.05$) levels of butyric acid and ammonia-N. Treatment with PL enhanced ($P < 0.05$) the crude protein preservation of silage by favoring the growth of *L. curvatus* and *Saccharomyces cerevisiae* and inhibition of lactic acid–assimilating yeast belonging to *Issatchenkia* during ensiling. In particular, treatment with PL advanced ($P < 0.05$) the productions of lactic acid and volatile fatty acid in IN-treated silage. Therefore, PL used as a new additive exhibited potential for improving silage fermentation when it is combined with LAB IN during ensiling.

Keywords: timothy silage, fermentation profile, phenyllactic acid, microbial community, high-throughput sequencing

INTRODUCTION

Timothy (*Phleum pratense* L.) is one of the most important cool season grasses grown mainly in cold regions of North America, Scandinavia, Russia, and Japan (Berg et al., 1996) and usually used for pasture, hay, and silages (Bélanger et al., 2001). On the basis of the high total annual yield and good feeding value, timothy is also introduced and cultivated on the Qinghai Tibetan Plateau. However, how to effectively preserve the nutrient of timothy silage is a challenge for local livestock producer in this region.

Under anaerobic condition, lactic acid bacteria (LAB) dominate the fermentation process for a sufficient pH decline from the productions of organic acids [mainly lactic acid (LA)] to preserve the nutrients of forage. In practice, many factors such as low ambient temperature and packing density lead to incomplete or poor silage fermentation on the Qinghai Tibetan Plateau (Li et al., 2019). Under this condition, some undesirable microorganisms such as lactate-assimilating yeasts, low-temperature–resistant bacteria, and/or clostridia robust and reduce the stability of silage, resulting in high dry matter (DM) and economic losses. Biological additives are available for improving the fermentation quality of silage in the cold region (Chen et al., 2020a). However, the variable

effectiveness from year to year is one of the main issues of using microbial inoculants (INs), because they are dependent on the environmental conditions and the forage characteristics (Muck et al., 2018). Therefore, chemical additives may be more effective in enhancing preservation of silage. Acids and/or their salts are the common active ingredients in chemical additives. The main acids used for silage additives are sorbic, benzoic, propionic, and acetic acids or their mixtures (Bernardes et al., 2015; Weiss et al., 2016). Commercial additives often contain mixtures of different acids at various concentrations to achieve the maximum effect against spoilage bacteria and fungi (Muck et al., 2018). Recently, some antimicrobial agents such as monopropionine and monobutyrin also showed a positive effect on silage preservation (Ferrero et al., 2019). In particular, propionate precursors such as fumaric, malic, citric, and succinic acids in the succinate-propionate pathway are applied in silage making (Guo et al., 2020).

Phenyllactic acid (PL) is an important broad-spectrum antimicrobial compound that inhibits the growth of undesirable microbes (most bacteria and some fungi) through multifaceted actions, even under low-temperature conditions (Rajanikar et al., 2021). PL is mainly derived from LAB genera such as *Lactobacillus*, *Pediococcus*, and *Weissella* (Bustos et al., 2018; Lipinska-Zubrycka et al., 2020; Wu et al., 2020). PL has been used in various bio-based materials in agricultural, pharmaceutical, and chemistry fields (Mu et al., 2009). In fact, PL is now well accepted as an alternative to antibiotics in livestock feeds (Nazareth et al., 2020). A study from Wu et al. (2020) has shown that PL could prevent crude protein (CP) degradation of alfalfa silage. However, limited information is available on how PL regulates microbiota for better silage preservation.

Hence, the aim of this study was to evaluate the effects of PL, LAB PL, and their mixture on the fermentation characteristics and microbial community composition of timothy silage on the Qinghai Tibetan Plateau. We hypothesized that PL was helpful for growth of LAB species during ensiling and subsequently advances silage fermentation of timothy in the cold region.

MATERIALS AND METHODS

Silage Preparation

This study was conducted on the Hongyuan Experimental Base of Sichuan Academy of Grassland Sciences (N 31°51′–33°33′, E 101°51′–103°22′, altitude 3,500 m, Hongyuan, P.R. China). Timothy was harvested at the heading stage, chopped at a length of 1–3 cm, and divided into 12 equal piles. In total, 3 of 12 piles were randomly assigned to one of the following treatments: (i) no additive as control (CK); (ii) PL [L815533, provided from Macklin Biochemical Technology Co., Ltd., Shanghai, China; at an optimal application rate of 10 mg/L fresh matter (FM) basis]; (iii) LAB IN (a mixture of *Lactobacillus plantarum* and *L. buchneri*, provided from Gaofuji Biological Technology Co., Ltd., Chengdu, China; at a recommended application rate of 10^5 cfu/g FM); (iv) PL + IN (PI). The LAB PL was diluted in sterilized water and applied using a hand sprayer, at a rate of 5 ml/kg of forage, by spraying uniformly onto the forage, which was constantly hand mixed. The PL was applied in a 500:1 w/w water-to-additive solution, using a hand sprayer, by spraying uniformly onto the forage, which was constantly hand mixed. The same amount of water was added to the CK treatment. The treated forage (about 50 kg) from each pile was divided into five equal parts. Each part (about 10 kg) was packed into a 20-L plastic bucket silo and sealed with a rubber gasket lid. All silages (n = 4 treatments × 5 storage time × 3 replications = 60) were stored in a dark room at ambient temperature (5∼15℃). Each treated silage was sampled after storage of 3, 7, 15, 30, and 60 days for determining the chemical composition and microbial community.

Chemical Analysis

Frozen samples of 20 g were mixed with 180 ml of distilled water for 3 min in a Stomacher blender. The pH of the filtrate was determined by a pH meter (PHSJ-4F, Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China). Filtrate of about 10 ml was subjected to centrifugation (4,500 × g, 15 min, 4°C), and the supernatant was analyzed for LA, acetic acid (AA), propionic acid (PA), and butyric acid (BA) using high-performance liquid chromatography (Li et al., 2019). Ammonia-N was determined by the method of Broderick and Kang (1980).

The DM content of each sample from silage at 60 days was determined by oven drying at 65°C for 48 h. Dried samples were ground through a 1 mm screen with a mill (DFY-300C, Linda Machinery Co., Ltd., Wenling, China). The CP of each sample was analyzed by the Kjeldahl method (AOAC, 1990). Neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were determined by the methods of Van Soest et al. (1991), using an fiber analyzer (ANKOM Technology, Fairport, NY), and expressed on a DM basis, including residual ash. When aNDF was measured, a heat-stable amylase (FAA, ANKOM Technology, Macedon, NY) was added following the instructions of the manufacturer. Water soluble carbohydrate (WSC) was determined by the method of Murphy (1958).

Microbial Analysis

The microbial population was determined by the method of Cai et al. (1999). Ten grams of each moist sample was put into a sterile glass bottle, suspended in 90 ml of saline solution, and shaken for 45 min in a laboratory blender (LB20ES, Shanghai Primesci Co., Ltd., Shanghai, China). Serial dilutions from 10^{-3} to 10^{-5} were produced. LAB were counted on (de Man Rogosa an Medium) MRS agar (GCM188, Land Bridge Technology Co., Ltd., Beijing, China). Plates of LAB were incubated at 37°C for 48 h in the anaerobic box (TEHER Hard Anaerobox, ANX-1; Hirosawa Ltd., Tokyo, Japan). Molds and yeasts were counted on Rose Bengal Agar with tetracycline (1.5 mg/L; YM01435, Shyuanmu Biomart Biotech Co., Ltd., Shanghai, China) and incubated at 30°C for 72 h. Yeasts were distinguished from molds on the basis of colony appearance and cell morphology.

Total bacterial DNA from each sample of silages was extracted using cetyltrimethyl ammonium bromide (CTAB) method. The PCR amplification and bioinformatic analysis of samples were

performed by the Novogene Bioinformatics Technology Co., Ltd., The primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1514R (5′- GNTACCTTGTTACGACTT-3′) were used to amplify the full-length 16S ribosomal RNA (rRNA) gene of the bacterial community. The primers (ITS9munngs TACACACCGCCCGTCG; ITS4ngsUni CCTSCSCTTANTDATATGC) were used to amplify the full-length ITS gene of the fungal community. The PCR reaction was conducted according to the description of Chen et al. (2020b). The purified PCR products were subject to generate libraries using SMRTbell^{TM} Template Pre Kit (PacBio). The high-quality libraries were sequenced on the PacBio Sequel platform. The quality filter, cluster, and analysis for 16S rRNA and internally transcribed spacer (ITS) sequencing data were performed as described by Chen et al. (2020b) and Pootakham et al. (2021). The data were analyzed on Novogene Magic Platform.[1]

Statistical Analysis

Data from silages were analyzed using the mixed procedure in SAS (v. 9.2). The following statistical model was applied: $Y_{ij} = \mu + S_i + I_j + S \times I_{ij} + e_{ijk}$, where the fixed effects were as follows: μ = overall mean, S_i = additive treatment, I_j = storage period, $S \times I_{ij}$ = interaction between additive treatment and storage period, and e_{ij} = error. The differences between means were assessed by Tukey's multiple comparison. The effect was considered significant when the probability was less than 0.05.

RESULTS

Chemical Composition, Fermentation Profile, and Microbial Population of Silages

The chemical composition of silage is shown in **Table 1**. Compared with CK, treatment with PL increased ($P < 0.05$) WSC content and decreased the NDF content of silage. The highest CP content and the lowest NDF content were observed in PI-treated silages. All additives increased ($P < 0.05$) LAB count and decreased ($P < 0.05$) yeast number of silage as compared with CK.

As shown in **Figure 1**, treatment with PL delayed ($P < 0.05$) the WSC consumption, whereas treatment with IN and PI advanced LA production to reduce the pH value during the first 30 days of ensiling. All treated silages showed a delayed ammonia-N production, especially for PI-treated silage. Treatments with IN and PI promoted the production of total volatile fatty acid during ensiling (**Figure 2**). Compared with CK, treatment with PL decreased ($P < 0.05$) the AA content of silage on the 60th day. In contrast to CK- and PL-treated silages, the IN- and PI-treated silages showed a decreasing trend in BA after 15 days of ensiling.

[1]https://magic.novogene.com

Bacterial and Fungal Alpha Diversities in Silage

The bacterial alpha diversity of silage is shown in **Table 2**. The observed species, ACE, and Shannon firstly increased and then decreased with prolonged ensilage time in CK silage. An opposite trend occurred to PL-and IN-treated silages. Compared with CK, additives increased bacterial alpha diversity indices of silage on the 7th day but decreased that of silage on the 30th day. In relative to PL-and IN-treated silages, low bacterial alpha diversity indices occurred to the PI-treated silage.

The fungal alpha diversity of silage is shown in **Table 3**. The indices of ACE and Shannon were increasing in CK- and IN-treated silages during ensiling. No differences in the indices of observed species, ACE, and Shannon of silages on the 30th day were observed between the CK and PL treatment. Compared with CK, treatments with PL and PI decreased fungal alpha diversity indices of silage on the 60th day. Notably, the highest values of observed species, ACE, and Shannon were found in IN-treated silage on the 60th day.

Bacterial and Fungal Community Compositions in Silage

The bacterial community composition of silage is illustrated in **Figure 3**. *Lactobacillus*, *Lactococcus*, and *Enterbacter* were the top three genera, with a total relative abundance of > 90%. The abundance of *Lactobacillus* increased in the CK silage during ensiling. High proportions of *Lactococcus* existed in PL-treated silage on the 60th day. In addition, treatment with PI facilitated the increase in abundance of *Enterobacter* in silage during ensiling. At species level, *L. curvatus* dominated in CK- and PL-treated silages during the first 30 days of ensiling, whereas *L. plantarum* dominated in IN-and PI-treated silages during ensiling.

The fungal community composition is illustrated in **Figure 4**. Most genera were unclassified fungi in silages. Prolonged ensilage increased abundance of *Saccharomyces*, *Rhizopus*, *Buckleyzyma*, *Issatchenkia*, and *Cladosporium allicinum*. Treatment with PL stirred the increase in abundance of *Saccharomyces* and decrease in *Rhizopus* and *Issatchenkia* in silage on the 60th day. At the species level, the abundance of *Rizopus* sp. and *Issatchenkia orientalis* increased in CK silage during ensiling. A similar trend occurred to *Saccharomyces cerevisiae* in PL-treated silage.

DISCUSSION

Chemical Composition, Fermentation Profile, and Microbial Population of Silages

Silage fermentation is driven by microbes (mainly LAB) under anaerobic conditions. Available substrates such as WSC are important for the propagation and growth of desirable microorganisms. In the present study, the WSC concentration (**Table 1**) was sufficient for initiating silage fermentation. However, low LAB counts and amounts of detrimental yeasts on the fresh plant were not beneficial for silage preservation. In fact,

TABLE 1 | Chemical composition and microbial population of fresh forage and silage on the 60th day.

Treatments	DM[II]	WSC	CP	aNDF	ADF	LAB	Yeasts	Molds
	% FW	%DM				Log_{10} cfu/g FM		
Fresh forage								
	26.43	8.61	11.23	57.59	34.06	2.78	4.33	3.06
Silage[I]								
CK	24.43	0.59^b	8.66^b	68.88^a	38.19	6.67^d	5.21^a	< 2.0
PL	24.31	1.52^a	8.82^b	64.11^{bc}	38.06	8.00^c	3.37^b	<2.0
IN	25.15	0.71^b	8.94^b	67.29^{ab}	37.13	8.95^a	3.15^c	< 2.0
PI	24.60	0.67^b	10.46^a	61.53^c	36.70	8.34^b	2.94^c	<2.0
SEM	0.19	0.12	0.23	0.71	0.40	0.02	0.21	< 2.0
P-value	0.445	< 0.001	<0.001	0.007	0.539	<0.03	<0.001	–

[a–c]Means within a row differed at level of $P < 0.05$.
[I]Silages were treated without (CK) or with phenyllactic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of L. plantarm and L. buchneri, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI).
[II]ADF, acid detergent fiber; CP, crude protein; DM, dry matter; FM, fresh matter; LAB, lactic acid bacteria; NDF, neutral detergent fiber; WSC, water-soluble carbohydrates; SEM, standard error of the mean.

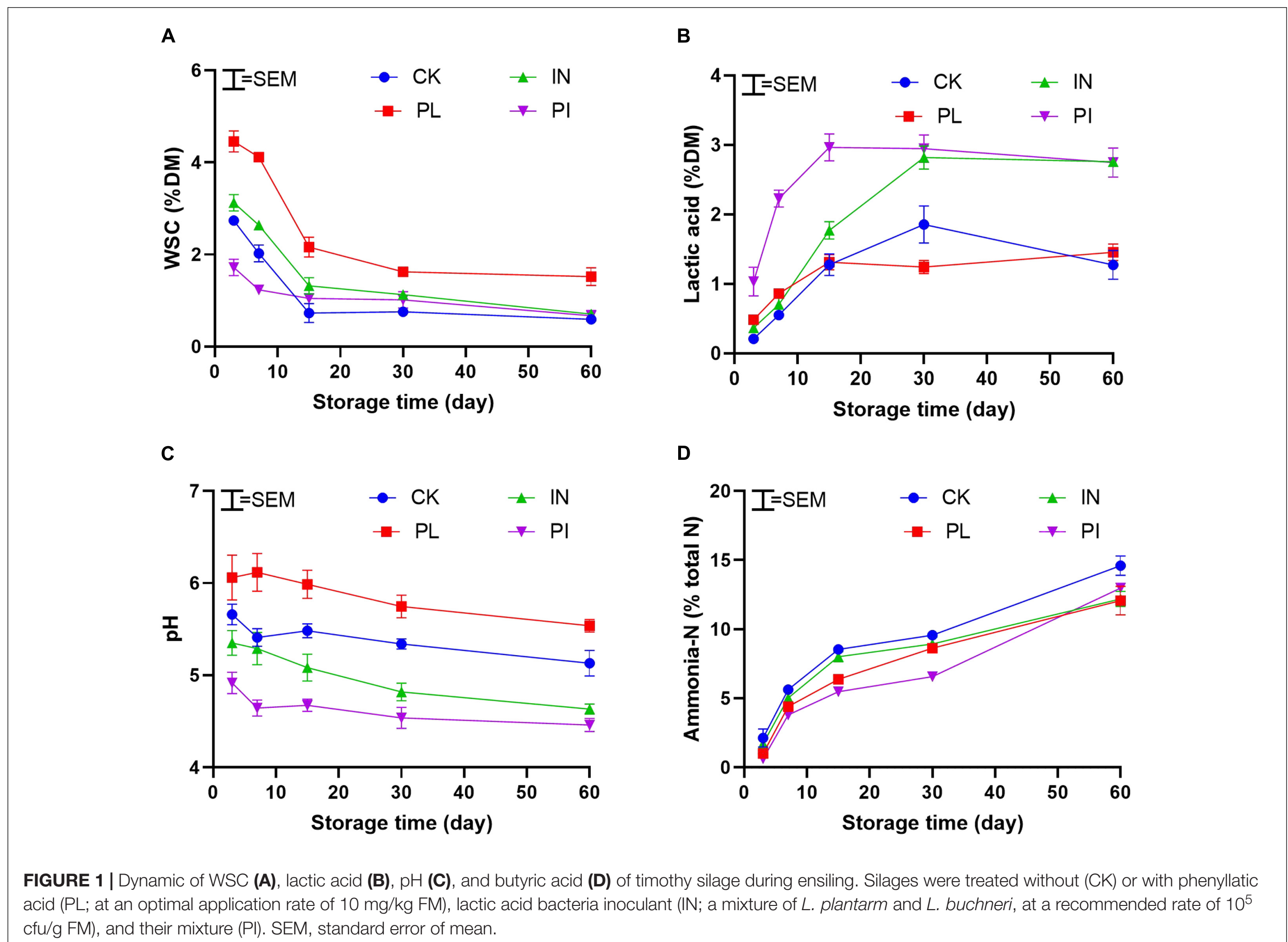

FIGURE 1 | Dynamic of WSC **(A)**, lactic acid **(B)**, pH **(C)**, and butyric acid **(D)** of timothy silage during ensiling. Silages were treated without (CK) or with phenyllactic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of *L. plantarm* and *L. buchneri*, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI). SEM, standard error of mean.

the control silage showed poor fermentation characteristics with high levels of pH, ammonia-N, and BA (**Figures 1**, **2**).

Various strategies have been used to reduce nutrient loss of silage. During ensiling, CP is degrading gradually, as indicated by increasing ammonia-N formation and BA production. Microbial PLs are recognized as important and widely used additives to improve the fermentation quality of silage. In the present study, however, the inoculation of LAB did not result in

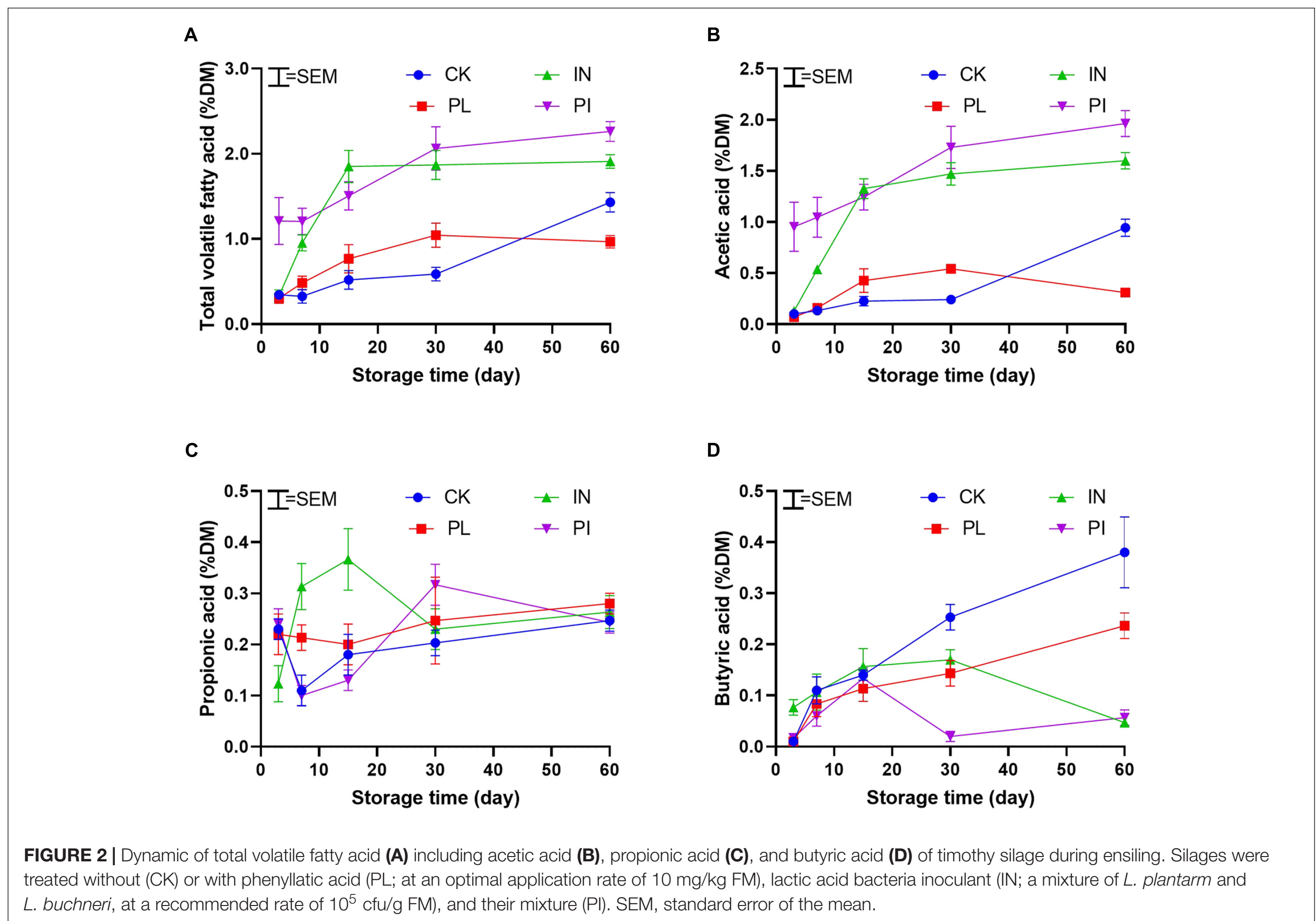

FIGURE 2 | Dynamic of total volatile fatty acid **(A)** including acetic acid **(B)**, propionic acid **(C)**, and butyric acid **(D)** of timothy silage during ensiling. Silages were treated without (CK) or with phenyllactic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of *L. plantarm* and *L. buchneri*, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI). SEM, standard error of the mean.

TABLE 2 | The alpha-diversity of bacterial community of silage samples.

Samples[I]	PE reads	Observed species	ACE	Shannon	Coverage
CK7[II]	6,244	21	21.45	0.58	0.999
CK30	10,616	50	54.71	2.73	0.993
CK60	12,689	31	32.05	1.23	0.993
PL7	11,563	43	46.54	1.74	0.983
PL30	4,668	24	24.43	0.68	0.996
PL60	7,929	32	40.22	1.19	0.989
IN7	11,623	36	48.16	1.39	0.997
IN30	14,085	32	33.59	0.81	0.992
IN60	11,601	39	40.02	2.30	0.994
PI7	12,114	33	33.44	0.83	0.992
PI30	12,536	25	29.48	1.10	0.996
PI60	9,899	16	21.19	0.43	0.996

[I]*Samples from silages treated without (CK) or with phenyllactic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of L. plantarm and L. buchneri, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI).*
[II]*Silages were sampled at days 7, 30, and 60 of ensiling, respectively.*

TABLE 3 | The alpha-diversity of the fungal community of silage samples.

Samples[I]	PE reads	Observed species	ACE	Shannon	Coverage
CK7[II]	8,546	21	29.53	1.62	0.997
CK30	9,506	50	65.23	2.85	0.992
CK60	9,522	50	88.60	3.00	0.989
PL7	10,363	27	74.78	1.79	0.993
PL30	8,323	50	62.06	2.91	0.992
PL60	9,609	33	65.12	2.16	0.992
IN7	5,718	30	43.86	1.55	0.997
IN30	6,663	45	59.58	1.79	0.992
IN60	3,913	117	174.19	3.78	0.977
PI7	8,372	29	38.62	1.69	0.996
PI30	9,068	34	64.73	2.43	0.992
PI60	8,371	43	44.75	2.45	0.998

[I]*Samples from silages treated without (CK) or with phenyllactic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of L. plantarm and L. buchneri, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI).*
[II]*Silages were sampled at days 7, 30, and 60 of ensiling, respectively.*

a high CP level in silage (**Table 1**). Our previous study has proved that the effectiveness of LAB inoculation varied between forage resources, management practice, and storage conditions (Chen et al., 2020a). PL has been applied in feed diets for improving animal performance. A study from Wu et al. (2020) has proved that PL exhibited a positive effect on

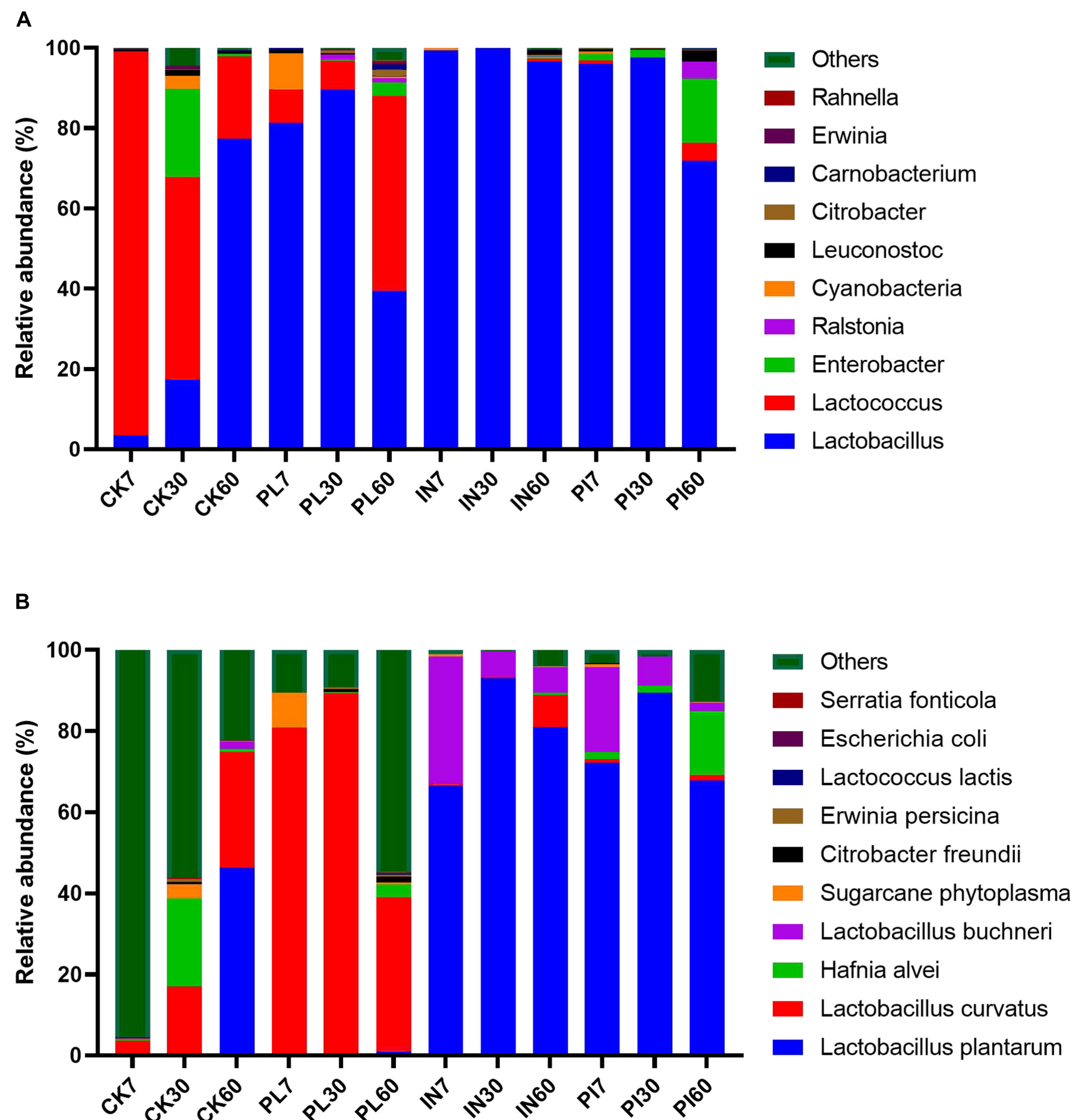

FIGURE 3 | Relative abundance of top 10 genera **(A)** and species **(B)** of the bacterial community in timothy silage. Silages were treated without (CK) or with phenyllatic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of *L. plantarm* and *L. buchneri*, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI).

the CP preservation of alfalfa silage. This situation was just observed in PI-treated silage. In addition, PL decreased the WSC consumption during ensiling, with a low fiber (NDF and ADF) concentration in silage. These indicated that a combination of PL and LAB could enhance the preservation of silage nutrients.

The preservation of silage nutrients was dependent on the fermentation rate and extent. The control silage exhibited a low production of LA and reduction of pH during silage fermentation (**Figure 1**). Most LAB PLs showed poor performance in silage production in cold regions because strains in commercial PLs are selected in temperate regions. In the present study, however, inoculation of LAB advanced the pH decline by quickly yielding LA during ensilage. A similar result was from Chen et al. (2020a,b) who reported that the use of LAB PL could advance the fermentation process of alfalfa and oat silages on the Qinghai Tibetan Plateau. AA is the active ingredient for controlling undesirable microorganisms such as yeasts in silage after exposure to air. Most commercial PLs contain hetero- and homo-fermentative LAB species, which could stabilize silage with the production of the most volatile fatty acids such as AA and PA. Thus, the AA in LAB-inoculated silage accumulated also at an early stage of ensiling (**Figure 2**). However, the AA concentration sustained stable after 30 days of ensiling. This may be attributable to how the species of AA-producing bacteria

exhibited low activity under a low temperature of < 15℃ (Zhou et al., 2016). PL is considered a natural environmentally friendly organic acid. The effect of PL on the fermentation characteristics of silage is controversial. Xu et al. (2019) found that PL had no significant effect on the fermentation products in whole-crop corn silage. A similar result was from Jung et al. (2019) who reported that PL level was not consistent with LA production in kimchi fermentation. Wu et al. (2020) used PL as an additive at ensiling and found that PL could promote the production of LA, AA, and PA in alfalfa silage. In the present study, the use of PL at ensiling did not enhance the fermentation performance of timothy silage. However, a synergistic effect of PL and LAB PL to effective advance productions of LA and total volatile fatty acid (mainly AA) for quick pH decline at an early stage of ensiling (**Figures 1**, **2**). The behind mechanism is worth exploring.

Bacterial and Fungal Alpha Diversities in Silage

As described previously, epiphytic microbiota regulated the fermentation rate and extent of silage. In turn, the microbial community was shaped by fermentation products. In the present study, the diversity (Shannon) and richness (ACE) indices of the bacterial community in control silage firstly increased and then decreased with the prolonged ensilage time (**Table 2**). Similar to our finding, an increase in bacterial diversity and richness indices occurred in barley, oat, triticale, and intercrop silages with relatively high pH levels (Duniere et al., 2017). However, the diversity and richness decline because of the continuous pH reduction from the accumulation of fermentation acids during ensiling (Polley et al., 2007). Ogunade et al. (2018) revealed that silage inoculated with LAB had decreased bacterial diversity indices due to the increased relative abundance of the predominant genus of *Lactobacillus*. This partly explained the decreased bacterial alpha-diversity of LAB-inoculated silage at the early stage of ensiling. However, the low temperature inhibited the fermentation of LAB-inoculated silage, resulting in a higher pH value, which provided conditions for undesirable microorganisms and subsequently increased the bacterial diversity (Chen et al., 2020b). The PL-treated silage showed a similar trend in bacterial alpha diversity. On the basis of weak acid theory, PL effectively inactivated most bacteria (Rajanikar et al., 2021), thus quickly reducing the diversity and richness of the bacterial community. When the silage continued to ferment for a lower pH value, the inhibitory effect was reduced, and the spoilage microorganisms robust again at the final stage of ensiling. Notably, a desirable situation in linearly decreasing diversity and richness of bacterial community during ensiling was found in PI-treated silage, indicating that the combinatory effect from PL and LAB was desirable.

Researchers pay limited attention to the fungal community in well-fermented silages, due to the absence of toxin-producing fungi (Duniere et al., 2017). The diversity and richness of the fungal community were increasing in CK silage during ensiling (**Table 3**). A similar result was found by Vu et al. (2019) who reported that the fungal community richness increased in elephant grass silage under natural conditions. Furthermore, this increasing trend was enhanced by the inoculation of LAB. Liu et al. (2019) reported that the fungal diversity and richness increased in LAB inoculated silage during ensiling. However, inconsistent results came from Keshri et al. (2018) who reported a remarkable decline in fungal Chao 1 index due to acidic and anaerobic conditions developed in *L. plantarum*–treated corn silage during ensiling. Research from Lavermicocca et al. (2003) demonstrated that the addition of LA could cause a 30% increase in the inhibitory activity of PL against fungi. Thus, the positive effect from the combination of PL and LAB on reducing diversity and richness of fungal community was observed in silage on the 60th day.

Bacterial and Fungal Community Compositions in Silage

The diversity and richness of microbial community were negatively correlated with the dominance of functional genera or species. Various microbial communities and succession were found in different silages (Parvin et al., 2010), and it is necessary to know the microbial community compositions to understand the complex process of ensiling (Xu et al., 2019). In the present study, *Lactobacillus*, *Lactococcus*, and *Enterbacter* were the top three genera, with a total relative abundance of > 90% (**Figure 3**). Studies from Cai et al. (1998) reported that *cocci* such *Lactococcus* prevail at the early stage of ensiling and were replaced by low-pH–resistant rods such as *Lactobacillus* species in silage. This situation was confirmed well in the present study because the ratio of epiphytic *Lactobacillus*/*Lactococcus* in control silage was increasing as the ensilage time prolonged (**Figure 3**). Inoculation of LAB could enhance silage fermentation by dominating the *Lactobacillus*. PL also advanced the dominance of *Lactobacillus*, but this positive effect was reduced at the late stage of ensiling, with high proportions of *Lactococcus* in silage at 60th day. At the species level, *L. curvatus*, *L. plantarum*, and *L. buchneri* dominated in silage. *L. curvatus* is mainly present in fermented foods. In this study, PL facilitated the growth of *L. curvatus* and extensively promoted the disappearance of inherent *L. plantarum* in untreated silage. However, PL exerted a positive effect on the dominance of *L. plantarum* in IN-treated silage. The behind reason is unknown. In addition, the relative abundance of *L. buchneri* in LAB-treated silage showed a decreasing trend during storage. Similarly, Zhou et al. (2016) and Chen et al. (2020b) reported that *L. buchneri* was disappeared or undetectable in corn, oat, and alfalfa silages under low temperatures.

The dominant fungal genera in silage was unclassified fungi (**Figure 4**). This was not in accordance with other results. The majority of fungi reported by Liu et al. (2019) in barley silages were *Issatchenkia*, *Cladosporium*, and *Alternaria*. Bai et al. (2020) found that unclassified fungi *Sporormiaceae*, *Ascochyta*, and *Candida* dominated in untreated-alfalfa silage. Genera of *Kazachstania*, *Cadida*, and *Picha* were heavily presented in natural-fermented sugar top silage (Wang et al., 2020). Many factors such as forage types and ambient storage temperature resulted in the discrepancy in fungal community composition. During ensiling, the total abundance of *Saccharomyces*, *Rhizopus*,

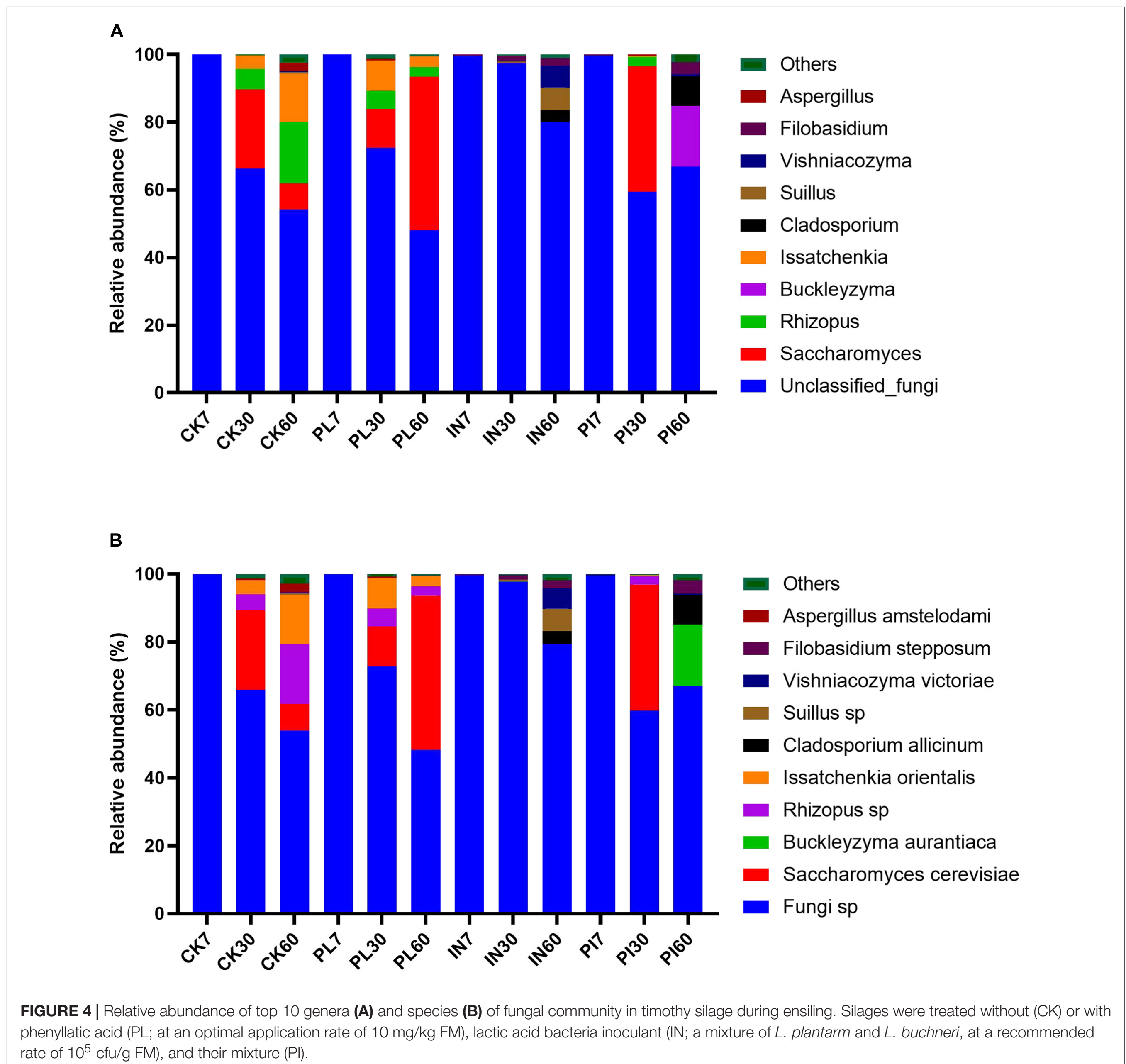

FIGURE 4 | Relative abundance of top 10 genera **(A)** and species **(B)** of fungal community in timothy silage during ensiling. Silages were treated without (CK) or with phenyllatic acid (PL; at an optimal application rate of 10 mg/kg FM), lactic acid bacteria inoculant (IN; a mixture of *L. plantarm* and *L. buchneri*, at a recommended rate of 10^5 cfu/g FM), and their mixture (PI).

and *Issyatchenkia* was increasing in control and PL-treated silages; *Cladosporium*, *Suillus*, and *Vishniacozyma* in PI-treated silages; and *Buckleyzyma*, *Cladosporium*, and *Filobasidium* in PI-treated silages. This indicated that the use of additives stirred the changes in fungal community compositions. The high distribution of yeasts belonging to *Issyatchenkia* is an indicator for the low aerobic stability of silage. The succession of fungal species in silage upon aerobic exposure is typically initiated by yeasts with the increase in pH, thereby allowing the low number of acid-tolerant spoilage microorganisms to proliferate (McAllister et al., 1995). *Saccharomyces cerevisiae* is considered a non-spoilage yeast because it does not assimilate LA and prevails in ryegrass, oat, barley, and sugar top silages (McAllister et al., 2018). According to the fact that the high abundance of LA-assimilating *Issyatchenkia* species decreased the aerobic stability of silages after exposure to air (Liu et al., 2019), thereby the addition of phenyllatic acid in timothy silage exerted a positive response to delay aerobic deterioration evoked by the proliferation of spoilage fungi. *Cladosporium*, as a producer of mycotoxins, is a member of the *Davidiellaceae* family and a ubiquitous mold (Tabuc et al., 2011). The presence of *Cladosporium* indicated that inoculation of LAB showed a limited effect on the stability of silage at the final stage of ensiling. Genera of *Rhizopus* occurred rarely in silages (Rodríguez-Blanco et al., 2021). It was first reported that *Buckleyzyma salicina* distributed in timothy silage.

CONCLUSION

Natural-fermented timothy silage showed poor fermentation on the Qinghai Tibetan Plateau. The use of PL could facilitate the growth of *L.* curvatus for better preservation of silage nutrients with low levels of ammonia-N and BA. The application of PL also showed a positive effect on the rapid production of LA and volatile fatty acid in LAB-inoculated silage. However, all silages exhibited potential for aerobic deterioration due to the high presence of undesirable fungi such as *Saccharomyces*, *Cladosporium*, and/or *Issatchenkia*. Further study is still needed to investigate the mechanism of reduced antifungal activity of PL during silage fermentation under low-temperature conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

PL and CC: conception and design of study and critical review and revision. YL, MZ, LC, and CZ: acquisition of data. PL and QC: analysis and interpretation of data. PL and YL: drafting the manuscript. QC and MZ: others. All authors read and contributed to the manuscript.

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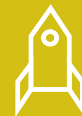

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