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The Effect of Different Lactic Acid Bacteria Inoculants on Silage Quality, Phenolic Acid Profiles, Bacterial Community and In Vitro Rumen Fermentation Characteristic of Whole Corn Silage

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Abstract: Corn silage is an important source of forage, but whether or not bacterial inoculants should be applied is somewhat controversial in ruminant feeding practice. In the present study, chopped whole corn plants treated with a single inoculant of *Lactobacillus buchneri* (LB), *Lactobacillus plantarum* (LP), *Pediococcus pentosaceus* (PP) served as either homofermentation (e.g., lactate only) or heterofermentation (e.g., lactate and acetate) controls and compared with those treated with either a mixture of the lactic acid bacteria (QA: 60% LP, 10%PP, 30% LB) or a mixture of the lactic acid bacteria (QB: 60% LP, 15% PP, 25% LB), to investigate their effects on the fermentation quality, ester-linked phenolic acids, and in vitro digestibility. After 60 day ensiling, the addition of QA exhibited the lowest pH (3.51) with greater lactic acid (LA) production. The ester-linked ferulic acid (FAest) and *p*-coumaric acid (pCAest) concentrations were significantly decreased during 60 days ensiling. And among all these groups, the LB and QA treated group showed a lower concentration of FAest and pCAest than other groups. After 60 days ensiling, *Lactobacillus* was the dominant genus in all LAB treated groups. Meanwhile, negative correlations of *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Prevotella*, *Ruminococcus*, and *Roseburia* with FAest content after 60 days ensiling occurred in the present study. *Komagataeibacter* was mainly found in LB and PP addition silages, and presented a significant negative effect with the level of acid detergent fiber (ADF). To explore whether the addition of LABs can improve digestibility of whole corn silage, an in vitro rumen fermentation was conducted using the 60 day ensiled whole corn silages as substrates. The QA addition group exhibited a greater 48 h and 96 h in vitro dry matter and ADF disappearance, greater 48 h gas production and less methane emissions. Even though there were the same neutral NDF levels in corn silages treated with LB and QA after 60 days ensiling, the QA treated silages with lower FAest and pCAest presented higher IVDMD after 96 h and 48 h in vitro fermentation. In brief, the addition of mixed inoculants of 60% LB,10% PP, 30% LB compared with the addition of whichever single HoLAB or HeLAB inoculants, facilitated the release of ester-linked phenolic acids (e.g., ferulic and *p*-coumaric acids) and remarkably, improved silage quality in terms of sharp pH decline and greater lactate production. Taken together with the improvement in rumen microbial fermentation, the results obtained in the present study provided concrete evidence for the role of mixed LAB application in corn silage preparation for ruminant feeding practices.

Keywords: whole corn silage; lactic acid bacteria; fermentation quality; microbial community; phenolic acid; in vitro fermentation

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1. Introduction

In ruminant feeding practice, ensiling is a traditional forage preservation method to improve the quality of feedstock which in turn often improves animal performance [1,2]. Whole corn silage is widely used in ruminant animals for its high nutritional value, good palatability, and comparatively low cost [3]. Plant cell walls are the major source of nutritional energy in the rumen of dairy cows, but less than 50% of cell wall fraction is readily digested and utilized by the ruminant host [3]. Many studies have already indicated that the structure and level of lignin and phenolic acids had a significant impediment to the digestion of the plant cell wall [4,5]. Ferulic and *p*-coumaric acid are the most abundant phenolic acids in plant cell walls, wherein they were reported to account for up to 80% of total phenolic acids [6]. In plant cell walls, ferulic and *p*-coumaric acid mainly link with arabinoxylans and lignin through ester and ether bonds [6]. These linkages were described as the molecular equivalent of spot welding on a steel mesh frame and played a dominant role in the inhibition of forage degradation [7]. These obstructions and the hydrophobicity of phenolic compounds hinder the access of hydrolytic enzymes to cell wall polysaccharides [8,9]. Grabber suggested that compared with the content of lignin, the ferulate cross-linkages had more important effects on the rate and extent of digestion of hemicellulose [10]. Thus, the release of FA and pCA from plant cell walls may increase the digestion of whole corn silage in the rumen.

Lactic acid bacteria (LAB) strains have been increasingly used to preserve the silage quality and reduce process-related losses for over 40 years [11]. However, whether or not bacterial inoculants should be applied is somewhat controversial in ruminant feeding practice. It is well known that the alteration of structural matrix in the plant cell wall can effectively increase the fiber digestion in ruminant animals. Different LABs were believed to take either homofermentation (e.g., lactate only) or heterofermentation (e.g., lactate and acetate) to promote silage fermentation, thus their combination may have alternative advantages or complementary effects [12]. Theoretically, during silage fermentation, the microbes and weak acid conditions might contribute to release part of the phenolic acids [13,14]. Li reported that after treatment of *Pennisetum sinese* silages with Acremonium cellulase, *Lactobacillus plantarum* A1 and Acremonium cellulase+ *Lactobacillus plantarum* A1, all groups noticed that ferulic acid could be released from plant cell walls [15]. However, a thorough understanding of different LABs on the conversion of pCAest and FAest during ensiling is still limited.

The objective of the present study was to attempt to elucidate the effect of different LABs in whole corn silages on the fermentation profile, phenolic acid composition and whether or not the LAB inoculation could improve in vitro rumen digestion and fermentation.

2. Materials and Methods

2.1. Experimental Design and Preparation of Corn Silage

A corn hybrid at a mature growth stage from the cropping field Qing Zhou city (Shan-Dong province, China) was harvested by a self-propelled silage harvester (Claas JAGUAR800, Beijing, China). The chemical composition of forage is shown in Table 1. The representative samples of whole corn plants were divided into 93 equivalent piles for the laboratory treatments. A total of three piles of samples of silage (approximately 500 g) were chosen at random and frozen at −20 °C as the initial silage for further analysis. The other 90 piles (5 microbial inoculation treatments × 6 ensiling duration × 3 replicates) were randomly assigned to the following treatments: (1) *Lactobacillus buchneri* JCBS-30 (LB), (2) *Lactobacillus plantarum* JYLP-376 (LP), (3) *Pediococcus pentosaceus* JYPP-16 (PP), (4) a mixture of the lactic acid bacteria (QA, 60% LP, 10% PP, 30 % LB), (5) a second mixture of the lactic acid bacteria (QB, 60% LP, 15% PP, 25% LB). All the LAB inoculants were evenly added at a concentration of 10^{11} CFU/g of fresh material. Briefly, the LABs were mixed with sterile distilled water as follows: 2 g of each LAB inoculant was diluted into 5 L sterile distilled water, to ensure the inoculum concentration was 2×10^5 CFU/g silage,

and sprayed onto the chopped corn plant material and evenly mixed. Afterwards, all the plant material was packaged into polyethylene plastic vacuum bags individually and then tightly vacuum-sealed using a vacuum packaging machine. Finally, all bags were taken back to the laboratory and stored at room temperature for 1, 3, 15, 30, 45 and 60 days, and each ensiling duration was arranged in three bag replicates.

Table 1. The chemical composition of whole corn plant before ensiling.

Item [1]	Value
DM	283 g/kg FM
WSC	117 g/kg DM
NDF	373 g/kg DM
ADF	214 g/kg DM
pCAest	9.54 g/kg DM
FAest	5.33 g/kg DM

[1] DM, dry matter; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; FAest, ester-linked ferulic acid; pCAest, ester-linked *p*-coumaric acid.

2.2. Sampling and Analytical Methods

A total of three replicates from each group, according to the designated ensiling time, were opened and sampled. A sample of 10 g fresh silage from each bag was homogenized in 90 mL distilled water in a juice extractor (JYL-C051 Jiu Yang juice extractor, China) for 90 s and filtered through four layers of filter paper. After the pH was determined, the filtrate was then centrifuged at 12,000 *g* (TG20M High-speed centrifuge, PingFan Instrumentation Co., Ltd., Changsha, China) for 15 min for further analysis of ammonia nitrogen (NH_3-N), water-soluble carbohydrates (WSC) and organic acids including lactic acid (LA), acetic acid (AA), propionic acid (PA) and butyric acid. The concentration of NH_3-N was measured by a microplate reader (RT-6500, Rayto Instruments, Shenzhen, China) following the method of Pang [16]. The determination of WSC was detected by microplate reader according to the anthrone colorimetry method described by Flsherisandf in 1973 [17]. The detection of organic acids used reverse-phase high-pressure liquid chromatography (Wufeng Co., Ltd., Shanghai, China) at a column temperature of 15 °C in which the wavelength was 210 nm. Separation was performed on a C_{18} column (5 µm, 250 mm × 4.6 mm, pH 2.0–8.0; Waters, Milford, MA, USA). The binary gradient solvent system contained in the detection of organic acids: (A) Chromatographic methanol and (B) 20 mmol NaH_2PO_4, adjusted to pH 2.5 with phosphoric acid. The flow rate of A to B is 14:86 *v*/*v*. The sample ran at a flow rate of 1 mL/min.

In order to determine chemical composition, silage samples were dried in a fan-assisted oven (DHG-9420A, YiHeng Scientific Instrument Limited Company, Shanghai, China) at 65 °C for 48 h and ground to pass through a 1 mm screen (KRT-34,KunJieYu Mechanical Equipment Co., Ltd., BeiJing, China). Samples were analyzed following the Association of Official Analytical Chemists [18]. Dry matter (DM) was analyzed in oven- drying at 105 °C for 4 h. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) content were determined according to Van Soest [19].

2.3. Determination of Extracted Phenolic Acids

The extraction of bound ferulic and p-coumaric acid followed the procedure of Cao's method [20]. Ester and total phenolic acids were released in 2.0 mol L^{-1} and 4.0 mol L^{-1} NaOH, respectively. The alkaline hydrolysis solutions were then acidified to a pH below 2.0 with H_3PO_4 overnight. Ethyl acetate was used to extract free phenolic acids from the pH-adjusted solution. Finally, the supernatant was evaporated under N_2 to dryness and resuspended in 1 mL methanol.

For the quantification of ferulic and *p*-coumaric acid content, 10 µL of extracted sample was injected into the HPLC. Separation was performed on a C18 column (250 × 4.6 mm, 5 µm, pH 2–8, Waters, Milford, MA, USA). The binary gradient solvent system contains (A)

chromatographic methanol and (B) formic acid in distilled water (0.4%, *v*/*v*). The gradient started at 36% solvent A, passing to 44% in 5 min, and back to 36% in 20 min. A 10µL sample was injected into the HPLC loop. The sample was run at 30 °C at a flow rate of 1mL/min at a wavelength of 320 nm. Ferulic and p-coumaric acid standards were purchased from Sigma–Aldrich Company (St. Louis, MO, USA) and dissolved in methanol to 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/mL. The standard curve was obtained by linear regression of peak area Y and target component concentration X under optimal chromatographic conditions as noted above.

2.4. In Vitro Rumen Fermentation

Three rumen-cannulated lactating Holstein dairy cows (4 years old, 60 ± 13 days in milk, body weight of 543 ± 45 kg and daily yield of 18.47 ± 0.77 kg) were housed in a free stall. The cows were fed daily 4.0 kg alfalfa hay, 4.0 kg whole corn silage and 6.0 kg commercial concentrate consisting of 500 g/kg corn meal, 150 g/kg wheat bran, 150 g/kg soybean meal, 50 g/kg rapeseed meal, 100 g/kg distillers dried grains, 10 g/kg limestone, 10 g/kg NaCl, 10 g/kg $CaHPO_4$, 10 g/kg sodium bicarbonate and 10 g/kg trace mineral and vitamin premix. Rumen fluids from each cow were collected 1 h before the morning feeding from different sites inside the rumen, squeezed through four layers of medical-use cheesecloth and mixed in equal proportions. The mixed rumen fluid was then transferred into a thermos pre-warmed at 39 °C and served as the inoculant for later batch culture. All the cows in our experiment were compliant with the Guidelines of Animal Care Committee and animal welfare guidelines of China Agricultural University (CAU20171014-1).

The in vitro digestibility of DM (IVDMD), NDF (IVNDFD), ADF (IVADFD) and kinetic gas production was followed by Pang's method [16]. A total of six replicates were performed for periods of 48 h for each sample to detect $IVDMD_{48}$, $IVNDFD_{48}$, and $IVADFD_{48}$. Additionally, three replicates were performed at 96 h to detect $IVDMD_{96.}$ Another five bottles without substrates served as blanks. A total of 700 mg of substrate was weighed into incubators. A volume of 50 mL of pre-warmed medium (pH 6.85) and 25 mL of strained rumen fluids were mixed into each incubator (glass bottle with a chitin plug, 125 mL total volume) [15]. Then N_2 was used to purge each bottle to remove air and bottles were sealed with Hungate's stoppers and screw caps immediately. All the bottles were connected with gas channel inlets of a Chinese-patented Automated Trace Gas Recording System (AGRS-III, China Agricultural University, Beijing, China), and fermentation gases were collected during a 48 h incubation with air bags for gas composition analysis [16]. After incubation at 39 °C for 48 h and 96 h, the bottles were removed from the incubation system and culture fluid pH was determined. To detect IVDMD, we filtered the biomass materials in every bottle with a nylon bag then dried it at 65 °C for 48 h. The residue was then used for further NDF and ADF analysis. Ammonia N and microbial protein (MCP) concentration were measured using the method of Verdouw and Makkar, using microplate reader 680 XR (Bio-rad, Hercules, CA, USA) [21,22]. The concentrations of volatile fatty acids (VFA) including acetate, ropionate, butyrate, and valerate, isobutyrate, isovalerate, isobutyrate acid, and isovalerate acid in the supernatants were measured by gas chromatography (GC522, Wufeng Instruments, Shanghai, China) following the method described by Chen in 2019 [23].

2.5. Bacterial Community Analysis

The extraction of genomic DNA was performed according to the procedure described by Wang [24]. A total of 10 g of silage was mixed with 40 mL of sterile 0.85% NaCl solution and then macerated in a shaker for 2 h. The samples were then filtered with sterile gauze. The filtrate was centrifuged (Pico-21, Thermo Fisher, Waltham, MA, USA) at 10,000 *g* and 4 °C for 15 min. The pellet was used to extract DNA. Total DNA was extracted using the E.Z.N.A™ Mag-Bind Soil DNA Kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer's instructions. The PCR reactions were performed in triplicate; 30 µL mixture, containing 1 µL of each primer. The PCR process was 3 min of

denaturation at 94 °C, 25 cycles of 30 s at 94 °C, 30 s for annealing at 45 °C, and 30 s for elongation at 65 °C, and a final extension at 72 °C for 5 min. The universal primer pair of 341-F: CCTACGGGNGGCWGCAG and 805R:GACTACHVGGGTWTCTAATCC was used to amplify the V3–V4 region. The PCR products were extracted from a 2% agarose gel. After purification and quantification, the PCR products were sequenced using Illumina Miseq™ (Shanghai Sangon Biotech Co. Ltd., Shanghai, China). Low quality sequences with quality scores below 20 were discarded.

Operational taxonomic units (OTUs) were clustered at a similarity level of 97% using Usearch (V11.0.667) [25]. Taxonomic classification for each OUT was determined with RDP Classifier (V2.12). The community composition of each sample was counted on domain, phylum, class, order, family, genus, species level. Rarefaction was performed on each sample to assess whether the sampling was adequate. The alpha-diversities of samples, mainly of the Shannon index, Simpson index, Chao index, and the Good's coverage, were created using Mothur software (V1.43.0). The correlation heatmap of the top tenth of the genera and fermentation properties were calculated using the gplots package in R.

2.6. Calculations

An exponential decay model was used to fit pH, LA, AA, WSC, and NH_3-N, using Equation (1):

$$Y = (Y0 - \text{Plateau}) \times e\,(-k * \text{time}) + \text{Plateau} \quad (1)$$

where time is the ensiling day, Y0 is the initial value of Y, plateau is the corresponding indicators k (day^{-1}) is the rate constant equal to the reciprocal of the time, time (day) is the ensilage day.

The Flieg's point index was calculated as Equation (2) [26]:

$$\text{Flieg}^{'}\text{s point} = 220 + 2 \times \text{DM} - 15 - 40 \times \text{pH} \quad (2)$$

where DM is the dry matter percentage in silage. Lower pH and higher DM content will result in a higher Flieg's point, implying the occurrence of a good quality ensiling process.

The cumulative gas production data were calculated as Equation (3) [27]:

$$GP_t = \frac{A}{1+\left(\frac{C}{\text{time}}\right)^B} \quad (3)$$

where GPt is the cumulative gas production (mL/g DM) at incubation time t (h), A is the asymptotic gas production (mL/g DM), B is a sharpness parameter determining the shape of the curve and C is the time (h) at which half of asymptotic cumulative gas volume is reached. A, B and C were calculated by the nonlinear procedure of SAS 9.4 (Cary, NC, USA).

Average gas production rate at the time when half of A occurred (AGPR, mL/h) was calculated with A, B and C using Equation (4):

$$\text{AGPR} = A \times B/(4 \times C) \quad (4)$$

We calculated the ratio of non-glucogenic to glucogenic acids (NGR) according to Orskov's method [28]:

$$\text{NGR} = (\text{Acetate} + 2 \times \text{butyrate} + \text{valerate})/(\text{Propionate} + \text{Valerate}) \quad (5)$$

where VFAs were expressed in molar proportions. Here, the non-glucogenic acids in ruminant animals refer to acetate and butyrate, also called lipogenic acids, which are primary acids for the synthesis of lipid in body tissue or milk. The glucogenic acids mainly refer to propionate which was absorbed to synthesize glucose through the glucogenic process in the liver. The appearance of valerate both in the numerator and denominator

stem from the fact that valerate may be expected, on oxidation, to yield 1 mole of acetic acid and 1 mole of propionic acid. Non-glucogenic acid included acetate, butyrate, and valerate acid. Glucogenic acids included propionate and valerate.

2.7. Statistical Analysis

Data were subjected to one-way analysis of variance using the general linear model (GLM) procedure of SAS V9.4 (SAS Institute, Cary, NC, USA). All data are presented as least squares mean. Least squares mean and standard error of mean (SEM) values were calculated with the least square means (LSMEANS) statement. Significance was declared at $p < 0.05$. Trends were recognized at $0.05 < p < 0.10$.

3. Results

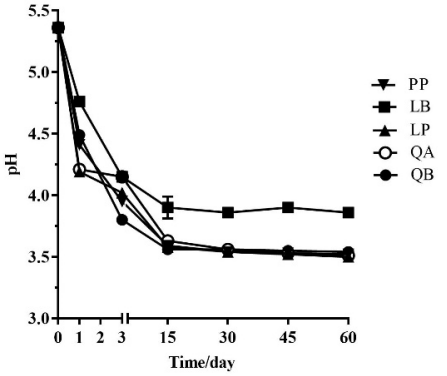
3.1. Fermentation Profile, Chemical Composition and Microbial Numbers of Silage during Fermentation

As shown in Table 2, pH, LA, AA, PA, WSC and NH_3-N in whole corn silages after 60 day ensiling were all affected by different microbial inoculants ($p < 0.01$). As shown in Figure 1, pH value in almost all microbial treated groups sharply declined to around 3.5 after 15 days ensiling, except that the LB group declined to around 3.8. The kinetic pH analysis in Figure 1A showed that the LP group presented the greatest rate of decrease in pH (k = 0.78), and all other groups presented relatively lower rates of decrease in pH (k = 0.54 to 0.65). Among five microbial treatment groups, the LB group presented the lowest LA content, and the highest LA occurred in the QA group at 30 days ensiling (74.61 g/kg DM). However, the kinetic parameters of LA showed that the greatest rate of in LA yield occurred in the PP group (k = 0.24). AA content in all microbial inoculant treatments showed a stepwise increase during the first 30 days ensiling and declined with subsequent silage fermentation. Among five treatment groups, both LP and PP groups presented lower AA yields, and the other group presented relatively higher AA yield as shown in Figure 1C. After 60 days ensiling, the lowest PA was in the PP group. Regarding short-chain volatile fatty acids, butyric acid in all groups was absent or detected in trace amounts. The Flieg's point was significantly increased in the 15 day fermentation and then reached a plateau (see Figure 1D). The addition of QA significantly increased Flieg's point more than other groups after 60 days ensiling. The WSC content in all treatment groups declined along with ensiling time. The LB group presented the lowest WSC content (7.86 g/kg DM) and the greatest NH_3-N content after 60 days ensiling (1.04 g/kg DM). The plateau values for WSC in PP-treated silage were the highest indicating high residual WSC in the PP-treated silage.

Table 2. Ensiling characteristics of whole corn silage after 60 days ensiling in response to different microbial inoculants.

Item [1]	Microbial Inoculant [2]					SEM [3]	*p* Value
	LB	LP	PP	QA	QB		
pH	3.85 ^{a}	3.50 ^{d}	3.52 ^{c}	3.51 ^{cd}	3.54 ^{b}	0.019	<0.01
Lactate (g/kg DM)	34.18 ^{c}	52.52 ^{a}	52.14 ^{a}	47.26 ^{b}	47.78 ^{b}	1.161	<0.01
Acetate (g/kg DM)	23.18 ^{a}	10.84 ^{d}	9.71 ^{d}	18.58 ^{b}	15.65 ^{c}	0.503	<0.01
Propionate (g/kg DM)	3.56 ^{c}	3.56 ^{c}	2.29 ^{a}	2.52 ^{b}	2.53 ^{b}	0.191	<0.01
Butyrate (g/kg DM)	0.01	0.01	0.01	0.01	0.01	0.004	ns
Lactae:Acetate	1.47 ^{e}	4.86 ^{b}	5.37 ^{a}	2.54 ^{d}	3.06 ^{c}	0.091	<0.01
WSC (g/kg DM)	7.86 ^{e}	24.72^{c}	44.61 ^{a}	38.42 ^{b}	15.73 ^{d}	0.787	<0.01
NH_3-N (g/kg DM)	1.04 ^{a}	0.84 ^{b}	0.87 ^{b}	0.52 ^{c}	0.81 ^{b}	0.012	<0.01

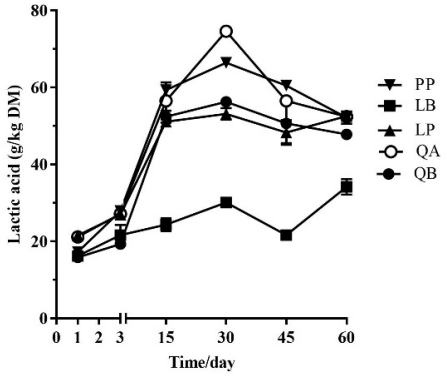
Means in a row without common superscript letter significantly differed as note *p* value. [1] DM, dry matter; LA, lactic acid; AA, acetic acid; PA, propionic acid; WSC, water-soluble carbohydrate; NH_3-N, ammonia nitrogen. [2] LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; PP, *Pediococcus pentosaceus*; QA, a mixture of lactic acid bacteria (60% LP + 10% PP + 30% LB); QB, a mixture of lactic acid bacteria (60% LP +15% PP + 25% LB). [3] SEM, standard error of least square means, n = 3.



(**A**)

Equal: Y = (Y0 − Plateau) * e$^{(-k*time)}$ + Plateau

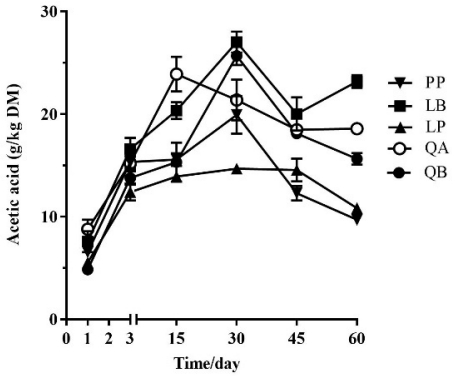
Value	LB	LP	PP	QA	QB
Y0	5.33	5.30	5.32	5.27	5.37
Plateau	3.88	3.57	3.55	3.59	3.55
k	0.54	0.78	0.61	0.64	0.65
R square	0.99	0.96	0.99	0.93	0.99



(**B**)

Equal: Y = (Y0 − Plateau) * e (−k*time) + Plateau

Value	LB	LP	PP	QA	QB
Y0	18.32	14.34	6.708	11.69	6.53
Plateau	31.44	51.87	60.19	59.51	52.24
k	0.037	0.16	0.19	0.16	0.16
R square	0.56	0.97	0.94	0.79	0.96



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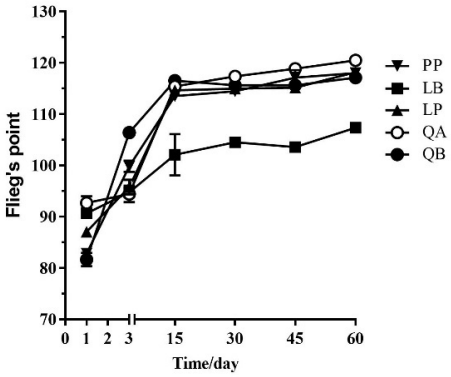

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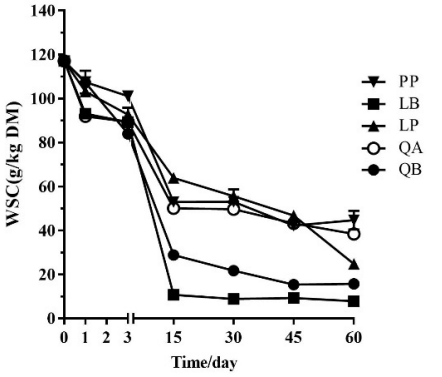
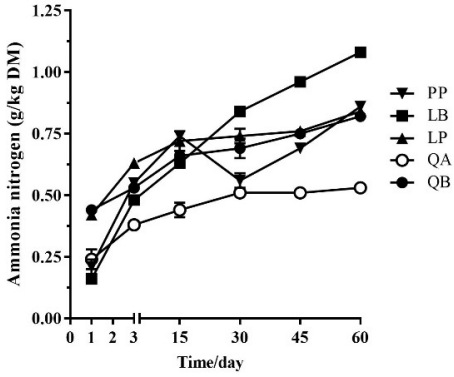


(**E**)

Equal: Y = (Y0 − Plateau) * e (−k * time) + Plateau

Value	LB	LP	PP	QA	QB
Y0	116.1	108.7	117.6	111.1	118.2
Plateau	6.45	26.66	44.66	43.51	16.61
k	0.14	0.04	0.11	0.15	0.13
R square	0.98	0.95	0.98	0.96	0.99



(**F**)

Equal: Y = (Y0 − Plateau) * e (−k * time) + Plateau

Value	LB	LP	PP	QA	QB
Y0	0.24	0.21	-0.17	0.12	0.45
Plateau	1.14	0.76	0.71	0.49	0.82
k	0.038	0.463	0.565	0.365	0.043
Rsquare	0.93	0.92	0.81	0.92	0.95

Figure 1. Effects of additives and ensiling time on pH (**A**), the concentrations of lactic acid (**B**), acetic acid (**C**), Flieg's point (**D**), water soluble carbohydrates (**E**) and ammonia nitrogen (**F**) of whole corn silages during ensiling.

3.2. Structural Carbohydrate Compositions of Whole Corn Silages after 60 Days Ensiling

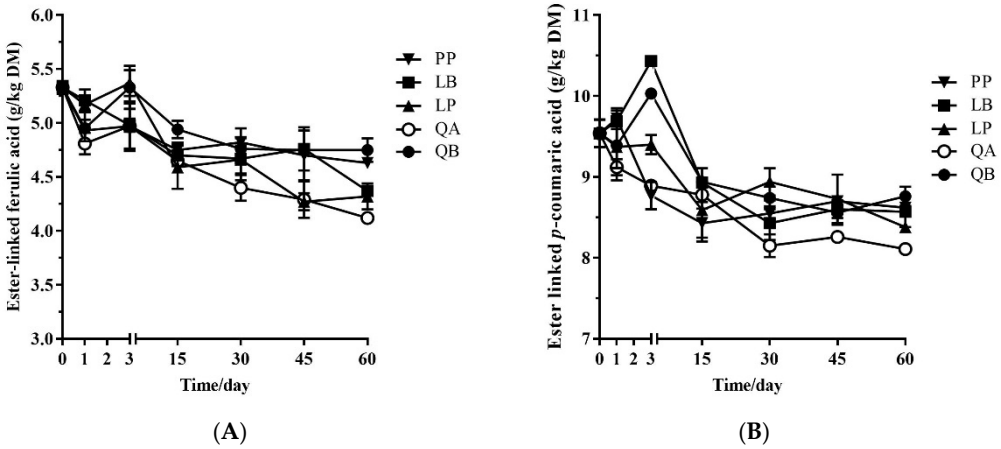
The chemical composition of whole corn silage in different LAB treated groups were significantly different as shown in Table 3. Compared with fresh whole corn plants prior to

ensiling, the DM of whole corn silage after 60 days ensilage showed a decrease, and the highest DM was observed in the LB and QA addition groups. LB and QA significantly reduced the contents of NDF compared with other LAB treated groups. The lowest ADF and hemicellulose levels were found in LB and QA, respectively. The content of FAest and pCAest were detected during ensiling (Figure 2). In the present study, the addition of LAB showed significantly different effects on the concentration of FAest and pCAest ($p < 0.01$). As shown in Table 3, the lowest FAest and pCAest were found after QA addition. As shown in Figure 2, the concentration of FAest was slightly decreased on the first day, but then FAest increased. After the first 3 days, the concentration of FAest decreased in all groups. The concentration of FAest reached a plateau after 15 days ensiling. The concentration of pCAest increased in the first 30 days then reached a plateau. The lowest FAest and pCAest were found after QA addition to whole corn silage, and the highest were in the QB group after 60 days ensiling.

Table 3. Structural carbohydrate compositions of whole corn silages treated with LAB and organic acids after 60 days ensiling.

Item [1]	Microbial Inoculant [2]					SEM [3]	*p* Value
	LB	LP	PP	QA	QB		
DM (g/kg FW)	283.08 [a]	265.67 [b]	268.44 [b]	280.02 [a]	269.27 [b]	2.259	0.007
Flieg's point	107.35 [c]	118.13 [b]	118.02 [b]	120.47 [a]	117.12 [b]	0.472	<0.001
NDF (g/kg DM)	347.28 [b]	368.95 [a]	368.97 [a]	345.13 [b]	378.33 [a]	4.905	0.01
ADF (g/kg DM)	196.49 [b]	215.83 [a]	203.32 [ab]	206.81 [ab]	215.44 [a]	4.104	0.001
Hemicellullose (g/kg DM)	150.78 [ab]	153.12 [ab]	165.64 [a]	138.31 [b]	162.88 [a]	6.093	0.002
Phenolic acid(g/kg DM)							
FAest	4.37 [b]	4.32 [b]	4.63 [ab]	4.12 [c]	4.75 [a]	0.160	0.002
pCA est	8.38 [b]	8.62 [ab]	8.11[c]	8.11 [c]	8.76 [a]	0.104	0.03

Means in a row without common superscript letter significantly differed as note *p* value. [1] DM, dry matter; FW, fresh weight; NDF, neutral detergent fiber; ADF, acid detergent fiber; FAest, ester-linked ferulic acid; pCAest, ester-linked *p*-coumaric acid; [2] LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; PP, *Pediococcus pentosaceus*; QA, a mixture of lactic acid bactria (60% LP + 10% PP + 30% LB); QB, a mixture of lactic acid bacteria (60% LP +15% PP + 25% LB). [3] SEM, standard error of means, n = 3.



(**A**) (**B**)

Figure 2. Effects of additive and ensiling time on ferulic acid and p-coumaric acid concentration of whole corn silage. (**A**) Dynamics of ester-linked ferulic acid; (**B**) Dynamics of ester-linked p-coumaric acid.

3.3. Bacterial Community of Silage after 60 Days Ensiling

As shown in Table 4, 913,158 quality-filtered sequences of 16S rRNA were acquired from 18 samples and clustered into 636 OTUs. The Good's coverage was above 99% in all treatments. Among all the silages, the addition of LP showed the lowest OUT numbers, Ace and Chao index ($p < 0.01$). The highest Shannon index was observed in the QA

addition group. Figure 3. indicated the relative abundance of bacterial composition in different LAB treated whole corn silage after 60 days ensiling. The dominant phylum is *Firmicutes* (69.43% to 97.23%) followed by *Proteobacteria* (0.79% to 25.52%) in all groups. The LP addition in whole corn silage presented the highest proportion of *Firmicutes*. The dominant family in all groups was *Lactobacillaceae* (61.67% to 90.67%). To gain further insight into the bacterial community of whole corn silage, microbial relative abundance at the genus level is presented in Figure 3C. *Lactobacillus* (59.23% to 89.62%) prevailed in all silage groups. The proportion of *Komagataeibacter* in LB and PP treated groups was 21.07% and 10.24%, respectively. The Pearson correlation coefficients between abundance of bacterial genera and fermentation indices of whole corn silage ensiled for 60 days are listed in Figure 4. A negative correlation was shown between NH_3-N and *Bacteroides*, *Blautia* and *Prevotella*. Positive correlations were observed between the relative abundance of *Bacillus*, *Bifidobacterium*, *Prevotella* and FAest. The concentration of pCAest was also negatively related to *Blautia.Bacteroides*, and *Lachnospiracea_incertae_sedis*.

Table 4. The α diversity of bacterial communities of different LAB in whole corn silage after 60 days ensiling.

Item [1]	Microbial Inoculant [2]					S.E.M [3]	*p* Value
	LB	LP	PP	QA	QB		
Reads number	58850	60847	48528	70558	46562	4133.1	ns
OTU[2] number	275.00 ^{b}	359.50 ^{a}	391.50 ^{a}	411.67 ^{a}	398.00 ^{a}	20.149	0.03
Ace	326.64 ^{b}	415.74 ^{a}	467.84 ^{a}	429.32 ^{a}	472.64 ^{a}	17.067	0.01
Shannon	1.81 ^{b}	0.61 ^{d}	1.26 ^{bc}	2.51 ^{a}	0.82 ^{cd}	0.078	0.01
Simpson	0.36 ^{c}	0.78 ^{a}	0.56 ^{b}	0.20 ^{c}	0.69 ^{ab}	0.02	<0.001
Chao	329.50 ^{b}	429.87 ^{a}	459.54 ^{a}	434.39 ^{a}	488.42 ^{a}	16.87	0.03
Coverage (%)	99.86	99.85	99.83	99.95	99.83	0.052	ns

Means in a row without common superscript letter significantly differed as note *p* value. [1] OTU, operational taxonomic units. [2] LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; PP, *Pediococcus pentosaceus*; QA, a mixture of the lactic acid bacteria (60% LP, 10%PP, 30%LB); QB, a mixture of lactic acid bacteria (60% LP +15% PP + 25% LB). [3] SEM, standard error of the difference of the means, n = 3.

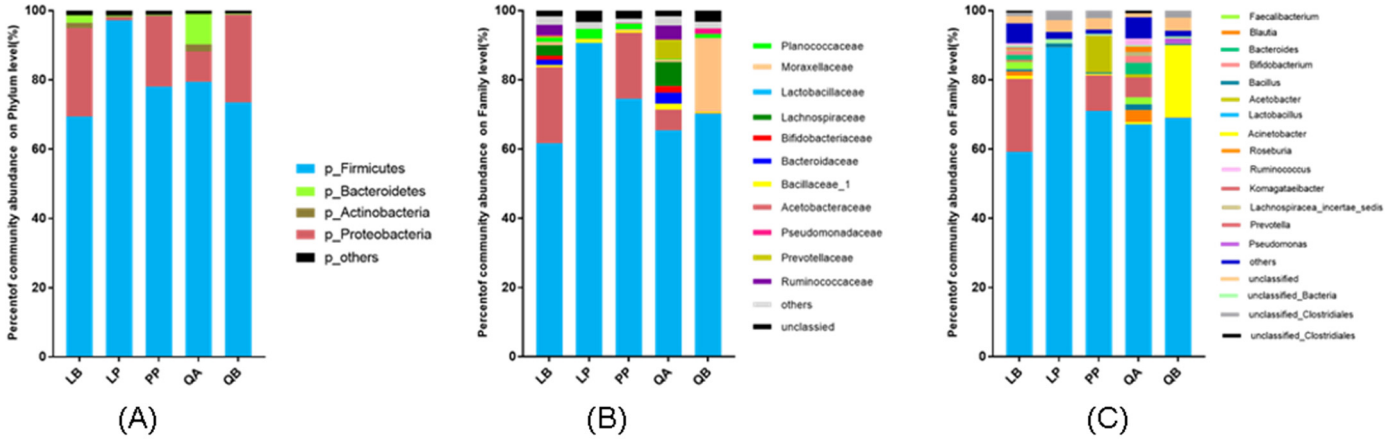


Figure 3. Bacterial community of whole corn silage after 60 days ensiling at the phylum (**A**), family (**B**) and genus (**C**) level, respectively.

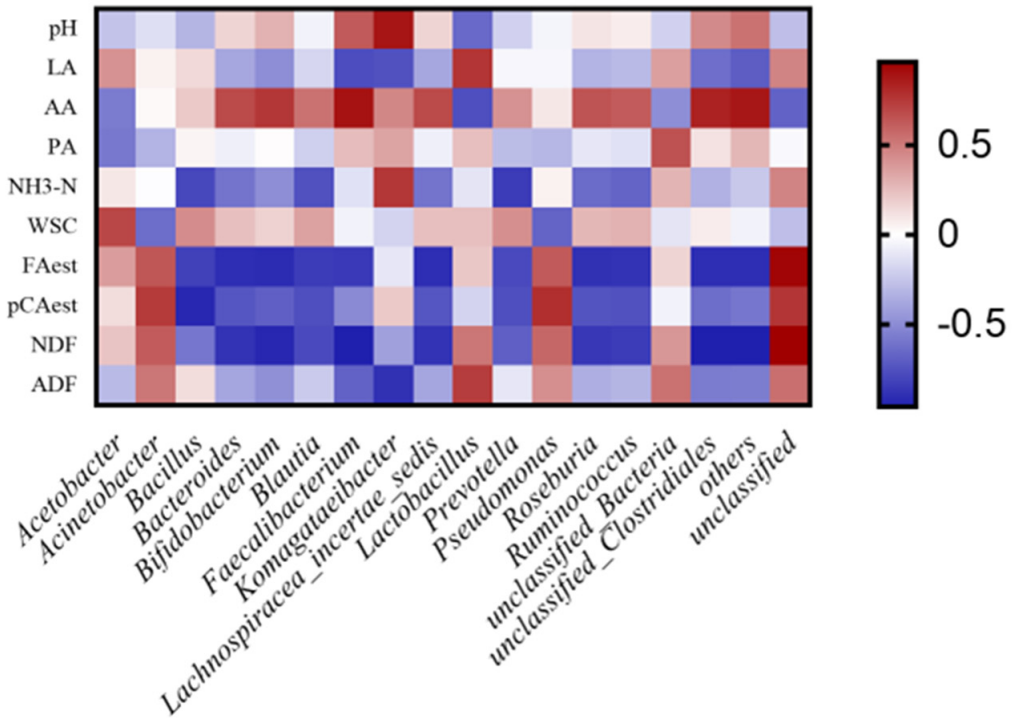


Figure 4. Spearman correlation heatmap of the top tenth of genera and fermentation properties. X and Y axis are environmental factors and genus. R in different colors to show, the right side of the legend is the color range of different *p* values.

LA, lactic acid; AA, acetic acid; PA, propionic acid; NH_3-N, ammonia nitrogen; ADF, acid detergent fiber; WSC, water-soluble carbohydrates; FAest, ester-linked ferulic acid; FM, fresh matter; NDF, neutral detergent fiber; NGR, ratio of non-glucogenic to glucogenic acids; pCAest, ester-linked *p*-comauric acid.

3.4. In Vitro Degradability and Kinetic Gas Production of 60 Days Ensiling Whole Corn Silage

Table 5 showed the in vitro degradability and kinetic gas production of 60 day ensilaged whole corn silage. QA presented higher 48 h, 96 h IVDMD, 48 h INVDFD and 48 h gas production than other groups ($p < 0.01$). Regarding gas production kinetics, the highest asymptotic gas production (A) and sharpness parameter determining the curve shape of the cumulative gas production (B) was noticed in QA and QB treated silage, respectively. Gas composition (e.g., H_2, CH_4, and CO_2) was listed in Table 5 and the lowest proportion of H2 and CH4 were presented in the QA addition group. LB addition to silage increased NH3-N in culture fluids and total VFA production more than other groups ($p < 0.01$). After 48 h fermentation, MCP in QA group was the highest.

Table 5. In vitro dry matter disappearance and kinetics of gas production of 60 day ensiling whole corn silage incubated with rumen fluids obtained from dairy cows.

Items [1]	Microbial Inoculant [2]					SEM [3]	*p* Value [4]
	LB	LP	PP	QA	QB		
Ruminal degradability							
$IVDMD_{48}$	77.84 ^{ab}	75.73 ^{c}	76.64 ^{bc}	78.48 ^{a}	76.04 ^{c}	0.465	0.02
$IVNDFD_{48}$	47.93 ^{a}	43.79 ^{b}	44.17 ^{b}	47.45 ^{a}	43.14 ^{b}	0.577	0.004
$IVADFD_{48}$	44.20 ^{bc}	45.55 ^{ab}	45.45 ^{ab}	46.55 ^{a}	42.42 ^{c}	0.643	<0.01
$IVDMD_{96}$	86.27 ^{bc}	87.15 ^{ab}	87.54 ^{ab}	88.20 ^{a}	85.23 ^{c}	0.532	<0.01
GP_{48}	79.35 ^{abc}	79.92 ^{abc}	71.30 ^{c}	88.98 ^{a}	75.46 ^{bc}	2.683	0.03
Gas production kinetics							
A (mL/g DM)	85.13 ^{ab}	81.81 ^{bc}	74.65 ^{c}	91.65 ^{a}	74.50 ^{c}	1.376	<0.01
B	1.93 ^{bc}	1.76 ^{c}	2.10 ^{ab}	1.76 ^{c}	2.17 ^{a}	0.059	0.01
C (h)	4.63 ^{c}	5.17 ^{b}	4.34 ^{d}	5.44 ^{a}	4.85 ^{c}	0.056	<0.01
AGPR	9.82 ^{a}	7.41 ^{c}	8.17 ^{bc}	8.19 ^{bc}	8.09 ^{bc}	0.262	<0.01
Ruminal fermentation profile							
pH	6.91	6.91	6.9	6.89	6.85	0.04	Ns
NH_3-N (mmol/L)	14.33 ^{a}	11.53 ^{c}	13.40 ^{ab}	12.65 ^{bc}	13.28 ^{ab}	0.34	<0.01
MCP (mg/mL)	0.42 ^{ab}	0.40 ^{b}	0.37 ^{b}	0.45 ^{a}	0.28 ^{c}	0.018	0.01
tVFA (mmol/L)	99.47 ^{a}	92.89 ^{b}	100.42 ^{a}	103.48 ^{a}	93.16 ^{b}	1.403	0.04
VFA pattern (%, molar)							
Acetate	48.42	48.55	48.93	48.60	47.95	0.337	ns
Propionate	36.73 ^{b}	37.42 ^{b}	36.40 ^{b}	38.27 ^{a}	36.56 ^{b}	0.37	0.02
Butyrate	10.53	10.52	10.81	10.59	10.83	0.286	ns
Iso-valerate acid	1.99	1.67	1.67	1.84	1.84	0.096	ns
Valerate	2.22	2.11	2.17	2.15	2.25	0.045	ns
Acetate:Propionate	1.31 ^{ab}	1.30 ^{ab}	1.36 ^{a}	1.25 ^{b}	1.35 ^{a}	0.024	<0.01
NGR	1.77 ^{b}	1.82 ^{b}	1.84 ^{b}	2.03 ^{a}	1.72 ^{b}	0.031	<0.01
Gas component (%, molar)							
H_2	0.06 ^{a}	0.05 ^{a}	0.03 ^{bc}	0.01^{c}	0.04 ^{ab}	0.006	<0.01
CH_4	16.57 ^{bc}	18.53 ^{a}	17.37 ^{b}	15.89 ^{c}	17.24 ^{b}	0.211	0.03
CO_2	83.35 ^{ab}	80.59 ^{c}	81.16 ^{b}	84.09 ^{a}	83.00 ^{ab}	0.664	0.01

Means in a row without common superscript letter significantly differed as note *p* value. [1] $IVDMD_{48}$, 48 h in vitro dry matter disappearance; $IVDMD_{96}$, 96 h in vitro dry matter disappearance. IVNDFD48, 48 h in vitro neutral detergent fiber degradability; IVADFD48, 48 h in vitro acid detergent fiber degradability; GP_{48}, cumulative gas production at 48 h; (A) asymptotic gas production, (B) sharpness parameter determining the curve shape of the cumulative gas production, (C) the time (h) at which half of A is reached; AGPR, average gas production rate when half of A occurred; MCP, microbial crude protein; [2] LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; PP, *Pediococcus pentosaceus*; QA, a mixture of lactic acid bacteria (60% LP + 10% PP + 30% LB); QB, a mixture of lactic acid bacteria (60% LP +15% PP + 25% LB). [3] SEM, standard error of means, n = 3. [4] NS, not significantly.

4. Discussion

4.1. Fermentation Profile, Chemical Composition and Microbial Profile of Whole Corn Silages during Fermentation

The pH and NH_3-N levels are direct indicators of silage quality. In this study, pH rapidly decreased and reached 4.15 within 3 days in all groups, which can depress the fermentation of *Clostridial* bacteria [29]. Organic acids including LA, AA, PA were measured to evaluate the fermentation quality of forage and were key factors to determine the pH during ensiling [30]. After 60 days ensiling, the concentration of LA in all treated groups (from 34.48 to 54.90 g/kg DM) was within the range of good quality silage (30 to 140 g/kg DM) [31]. According to the fermentation pattern, LABs can be classified into homofermentative (HoLAB, such as *P. pentosaceus*) and heterofermentative LAB (HeLAB, such as *L. buchneri*) [32,33]. *L. plantarum* is a facultatively heterofermentative lactic acid bacteria which could tend to homofermentation under sufficient WSC [34]. Homofermentative cultures could promote the production of LA which contributed to the highest decline of pH while HeLAB inhibits yeast and mold growth by producing high concentrations of AA [30]. In accordance with the tendency of pH, the concentration of LA increased rapidly in the first 15 days. The lowest pH and LA were found in the LB treated group which is an HeLAB. The addition of HeLAB tends to produce more AA which helps to improve aerobic stability [35]. Thus, higher AA in the LB treated group may indicate higher aerobic stability which was consistent with Zhang's research [36]. Addah suggested that silage

treated with *L. buchneri* could degrade the accumulated LA into AA, 1,2-propanediol, and ethanol while *L. plantarum* was able to degrade LA into formic acid [37]. This could explain the slight decrease in LA after 30 days ensiling. The production of PA was due to secondary fermentation which was carried out by *Clostridium, Bacillus or Propioni* bacteria [38]. Compared with other LAB groups, silages with *P. pentosaceus* lead to lower PA content. This result suggested that the PP addition could inhibit secondary fermentation. The QA and QB treated groups presented higher AA than HoLAB treated silage and higher LA than the HeLAB group. This result indicated the addition of mixed HeLAB and HoLAB lead to both homofermentation and heterofermentation in silage. During the ensiling process, LAB could metabolize WSC or some non-water-soluble hemicelluloses into organic acids, mainly LA, by which the growth of LAB can be accelerated [39]. In this study, the content of WSC decreased during the ensiling which was consistent with Dunière's study [40]. Compared with other groups, PP treated silages produced the greatest LA with the least WSC utilization after 60 days ensiling. During ensiling, the concentration of NH_3-N increased gradually which associated with *clostridial* and *enterobacteria* activity by decomposing protein in raw materials [41,42]. LAB additives showed potential to protect feed proteins in silage through a meta-analysis [8]. The rapid decrease in pH inhibited proteolysis caused by plant enzymes and reduced the rate of oxidation of NH_3-N into nitrites and nitrates which could further reduced the emission of obnoxious gases during ensiling [15,43]. In the present study, lower NH_3-N was found in HoLAB groups with lower pH. The quality of whole corn silage could be estimated based on Flieg's point [26]. This index was determined by pH and DM [44]. The highest Flieg's point in QA indicated that the addition of QA had better fermentation quality than other LAB addition groups. During ensiling, the Flieg's point increased in the first 15 days then remained stable.

4.2. Chemical Composition of Whole Corn Silage in Response to Different LAB after 60 Days Ensiling

The ensiling process significantly affected the chemical composition of silages.

Xie and Kleinschmit found a decrease in DM in corn and sorghum silages after silage ensiling which was also observed in the present study [45,46]. The NDF and ADF are important indexes of silages which could affect their utilization in ruminants. The present study noted that compared with fresh silage, the addition of LAB decreased the concentration of NDF and ADF after 60 days ensiling. These results indicated a positive effect of the inoculants during fiber degradation and were well in agreement with Li's and Zhao's studies that *L. plantarum* could reduce the content of ADF and NDF after 60 days ensiling [47,48]. However, some studies indicated that the addition of LAB could not decrease or even increase the concentration of NDF and ADF compared with the control. Compared with pre-ensiling material, hemicellulose presented a slight decrease in LB, LP, and QA treated silages. This result can be ascribed to the acid hydrolysis of hemicelluloses at the low pH value [49].

4.3. The Change of Ester-Linked Ferulic and P-Coumaric Acid of Whole Corn Silage during Ensiling in Response to Different LAB Inoculants

Ferulic acid in plant cell walls is ester-linked via its carboxylic acid group to the C(O)5 position of the arabinofuranosyl side group attached at the C(O)2 to the xylan chain [50]. The *p*-coumaric acid in plant cell walls mainly bound to lignin, only a small amount attached to polysaccharides [51]. These cross-linkages limited the access of rumen microbes and inhibited the attachment of microbial enzymes [9,52]. It is well documented that the hydrolysis of linkages between phenolic acids and plant cell walls could help to release part of the hemicellulose and lead to a higher digestibility of silage [15]. Therefore, it is presumed that the application of LABs could cleave ester bonds, which can be used as a parameter to characterize the degradation of lignocellulose by the addition of LAB. In the present study, the concentration of FAest and pCAest were increased in the first 3 days which was ascribed to the preferential utilization of nutrients such as WCS in the first 3 days. After the first 3 days, the concentrations of pCAest and FAest were decreased in all groups.

The silage sugarcane also showed a lower concentration of FAest after ensiling which is consistent with our results [53]. Thus, the ensiling process could decrease the concentration of FAest. However, in Xie's study compared with fresh sorghum plant, the ensilaged sorghum showed an increase in the concentration of both FAest and pCAest [45]. This might be explained by the utilization of nutrients during ensiling being preferential thus the concentration of FAest and pCAest were increased in the first 3 days. The increase in FAest and pCAest was noticed in the first 30 days and a slight decrease in pCA and FA was noticed in some groups after 30 days ensiling, which could be explained by some microbes during ensiling catabolizing ferulic and p-coumaric acid to 4-vinylguaiacol, dihydroferulic acid and other substrates [54].

The addition of different LABs showed different abilities to decrease the concentrations for extraction of phenolic acids. Compared with other groups, the QA addition group showed a lower concentration of FAest and pCAest after 60 days ensiling while the QB group showed the highest concentration of FAest. This result suggested that compared with other groups, QA addition released more ester-linked phenolic acids from whole corn plants. Li found an increase in free ferulic acid during the ensiling of *Pennisetum sinese* silages [15]. Xu et al. isolated several LABs (including *L. amylovorus CGMCC 11056*, *L. acidophilus CCTCC AB2010208*, *L. farciminis CCTCC AB2016237*, and *L. fermentum CCTCC AB2010204*) with FAE activities which could degrade hydroxycinnamic esters and release hydroxycinnamic acids from corn stover [55]. This research is consistent with our study that the ester-linked phenolic acids could be released from plant cell walls during ensiling. However, different kinds of LAB presented different abilities for the release of phenolic acids from plant cell walls. Li et alfound a higher ($p < 0.05$) free ferulic acid in an L. plantarum A1 treated group [15]. Xie et al also reported a higher FA and pCA extraction yield in LP23-treated sorghum silage than the control group at 30 °C [45]. All these results confirmed that the addition of QA and LP significantly improved the extraction of FAest and pCAest.

The authors in the present study also detected the ether-linked FA and pCA during ensiling. However, no significant change of ether-linked FA and pCA were detected. Thus, these data were not listed in the results tables of the present study. This could be explained by the previous conclusions that the ether linkages between ferulic acid and the lignin structure cannot be broken in anaerobic conditions [56]. The *p*-coumarate ethers are linked only to lignin and enzyme treatments on ether-linked pCA were ineffective [57,58].

According to these results, the authors believed that the inoculation of microbes during ensiling could break the structure of lignocellulose through hydrolyzation of FA ester linkages between lignin and hemicelluloses, and thereby increase the accessibility of microbes or enzymes. The release of phenolic acids reinforces the idea that LAB treated silage is a commercial way for the recovery of phenolic acids from plant cell walls and increasing the utilization of forages [55].

4.4. Bacterial Community of Silage after 60 Days Ensiling

The fermentation of silages takes place by a complex microbial community which is affected by environmental factors, such as climate, geographical location, and the additives used in silage [59,60]. The constituents of the bacterial community in the silage could somewhat explain the changes in fermentation characteristics. In the present study, the bacterial community showed a significant difference in response to the addition of LAB after 60 days ensilage. The bacterial community in whole corn silage after 60 days ensiling are in the phylum Firmicutes, mainly consisting of families *Lactobacillaceae*, *Planococcaceae*, and *Ruminococcaceae* and *Lactobacillus, Prevotella* and *Bacillu* in genera. The predominance of Firmicutes and Proteobacteria at the phylum level was also found in barley, grass, whole corn silage, and alfalfa silages in previous studies [35,60–62]. After 60 days ensiling, most undesirable microbes were inhibited and *Lactobacillus* occupied the main composition of microbes. *Lactobacillus* also was reported as the dominant genus in Ni 's study [63]. *Lactobacillus* could enhance LA production and reduce pH and was usually present during

ensiling [63]. Compared with HeLAB, the HoLAB treated silages showed higher abundance of *Lactobacillus.* A positive but not significant correlation was found between *Lactobacillus* and LA in the present study.

Acetobacter could metabolize sugars and alcohol to AA and potentially inhibit the growth of harmful microbes and thus improve the aerobic stability of silage [60].The high richness of *Acetobacter* in the PP group may imply a higher aerobic stability of silage. In some small-scale laboratory and farm bunker-silo corn silage, the dominance of *Acetobacter* also has been reported [64]. Negative correlations between *Bacillus*, *Bacteroides*, *Bifidobacterium*, *Blautia*, *Prevotella*, *Faecalibacterium*, *Lachnospiracea_incertae_sedis*, *Ruminococcus,* and *Roseburia* and the concentration of FAest after 60 days ensiling were found in this paper. This result suggested that these strains might have the attribute of releasing the linked-phenolic acids from plant cell walls. *Bifidobacterium* strains have been reported to produce FAE [65,66]. Several *Bacillus* spp. such as *Bacillus pumilus* W3, were found to have FAE activity [67]. *Ruminococcus albus* has been reported to release phenolic monomers from plant cell walls and cinnamoyl esterase activity was also found in the genus *Ruminococcus* [68]. The ability of these strains to produce FAE could explain the negative correlations between *Bifidobacterium*, *Bacillus*, *Ruminococcus*, and FAest in this research. Negative effects between *Lactobacillus*, *Bacteroides*, *Bifidobacterium*, *Faecalibacterium,* and NDF were found in this paper. The addition of LB and PP presented higher levels of *Komagataeibacter* and a significant negative effect was found between *Komagataeibacter* and ADF. *Komagataeibacter* with fibrolytic activity has been reported in many studies [2,69]. Thus, the author suggested that the different additions of LAB could affect fibrotic substances by shaping the pattern of microbial colonization.

4.5. In Vitro Rumen Fermentation Characteristic of Whole Corn Silage in Responses to Different LAB Inoculants

4.5.1. In Vitro Rumen Degradation in Different LAB Treated Whole Corn Silage

The digestibility of forage is one of the most crucial indexes which could directly reflect the quality of silage [47,60]. In the present study, a significant difference occurred for 48 h and 96 h IVDMD, 48 h IVNDFD, and 48 h IVADFD, and these results were consistent with Guo's results that *L. plantarum* and *E. faecalis* were able to increase IVDMD, and IVNDFD [49]. Previous studies have reported that the addition of LAB had a positive effect or no effect on fiber fraction and degradability [70,71]. In the present study, the addition of different LAB significantly affected the IVDMD, IVNDFD, and IVADFD. Nsereko noted that the inoculation of LAB together with FAE addition were effectively cultivated in silage on exposure to ruminal microbes and enzymes which enable silages to be more fermentable in the rumen [72]. Xie reported that there was no significant difference in NDFD between control and LAB-treated silages, and they speculated that the improvement of NDFD may be more dependent on other characteristics rather than the presence of FAE [45]. Mixed LAB inoculants containing *L. buchneri* LN4017, *L. plantarum,* and *L. casei* increased 24 h and 48 h ruminal in situ NDF digestibility [73]. The positive response of the LAB treated group could be ascribed to the breakdown of plant cell wall structures. The latter effectively released the intracellular contents and supplied more positions and substrate for rumen microbes to attach and degrade, resulting in the final rumen degradation improvement. The linkages between phenolic acids and plant cell walls could limit the utilization of feed. Some previous studies notedthe concentration of FAest in plant materials was positively correlated with NDF and ADF digestibility [56,57,74]. However, the FAest did not promote the utilization of nutrients, it was just accompanied by the utilization of nutrients. Thus, in this paper, the concentration of FAest did not have a negative correlation with the in vitro rumen digestibility. The *p*-coumaric acid in plant cell walls is mainly linked to lignin and limits the utilization of plant cell walls. Previous studies reported that some LABs (such as LB, LP) were able to produce FAE to breakdown the ester bonds and release side chains and make the polysaccharide backbone, consequently ruminal degradation was increased [75–77]. In this paper, the lowest pCAest was found in QA treated silage with the

highest IVNDFD. The concentration of pCAest was reported to have negative effects on digestibility in some studies [78]. Thus, the breakage of linkages in plant cell walls helps to increase the utilization of feeds in the rumen.

4.5.2. Kinetic Gas Production in Different LAB Treated Whole Corn Silage

Regarding kinetic gas production, asymptotic gas production (A) and cumulative in vitro gas production usually reflect the extent of rumen fermentation, and they were reported to be positive along with the rumen degradation rate [16]. The highest values of A and 48 h gas production corresponded with the highest IVDMD in the QA addition group, suggesting that QA treated whole corn silage had a beneficial effect on the fermentation potential. The gas production can reflect the fermentation status and the utilization of feed. Even though the NDF in the LP and PP groups was almost the same after 60 days ensiling, the GP_{48} was significantly different. This could be explained by the difference in contents of FAest and pCAest after ensiling [57]. With the linkages broken, the feed provided more sites for microbes to attach which led to higher gas production.

4.5.3. In Vitro Rumen-Fermentation Characteristic Responses to LAB

VFAs are the predominant end products of the rumen and are the main metabolizable energy for ruminants. The production and the proportions of VFA can somewhat reflect the metabolism status of rumen microorganisms to let us estimate whether the microflora in the rumen is predominantly fibrolytic or predominantly amylolytic [79] In this study, the highest total VFA was found in the QA group corresponding with a high IVDMD. Generally, a greater extent of substrate degradation was accompanied by more VFA production [23]. The above results implied that the addition of QA not only enhanced the digestion of whole corn silage in rumen, but also sped up the metabolism rate of digested nutrients resulting in VFA production. In the present study, the highest PA production and IVNDFD found in QA treated silage were consistent with previous studies showing that the increase in NDF digestibility of cattle fed inoculated silage was positively associated with increased ruminal PA [73]. It is reported that the synthesis of acetate resulted in a greater volume of gas production than propionate synthesis [75]. Chen reported that the addition of LAB (*L. plantarum* and *P. pentosaceus* combination) increased PA in rumen [80]. Guo also reported that the addition of *L. plantarum* and *Enterococcus faecalis* in corn stover silage could increase the propionate and decrease acetate/propionate in an in vitro study [49].

In the rumen, protein or non-protein nitrogen are degraded into ammonia-N which can be revitalized with peptide and amino acids by microbes to synthesize microbial proteins (MCP) [23,81]. Therefore, MCP could somewhat reflect the growth rate and population of microbial cells [23]. In the present study, greater MCP production in the QA and LB groups might indirectly imply the addition of LAB inoculant was beneficial to the growth of rumen microbes.

4.5.4. Fermentation Gas Composition

Methane and carbon dioxide emissions are greenhouse gases produced during enteric fermentation in the ruminant digestive tract. The gas composition during in vitro rumen fermentation was affected by the addition of LAB in whole corn silages. The reduction in methane emissions in LAB treated silage have also been reported in other studies. The addition of LAB reduced the rumen methane emission also reported in grass silage, sorghum silage and high moisture alfalfa silage [82,83]. Addah suggested that the corn silage treated FAE-producing inoculant could also reduce the enteric methane emissions [43]. However, through an in vivo study, Holstein Friesian dairy cattle showed no significant change in CH_4 emission in both long-and short term LAB treated silage [84]. These studies were consistent with our results that different LAB may present different effects on the rumen fermentation gas composition.

The different methane output during rumen fermentation is strongly related to forage quality and NDF digestibility [85]. Addah noted that the increase in ruminal fiber digestion

lead to a rise in ruminal nutrient digestion, fermentable substrates, and the passage rate which could reduce time available for the production of methane in the rumen [43]. In the present study, the lowest CH_4 emission was noticed in QA with the highest IVNDFD, which agreed with Addah's conception [43]. VFA fermentation patterns of fiber digestion also influenced the output of CH_4 [43]. Propionate formation is an alternative to hydrogen formation which can decrease the substrates for methane production [86]. In the present study, the addition of QA showed the highest PA concentration with the lowest CH_4 after 48 h rumen fermentation. According to the present results, the authors noticed a glucogenic pattern of rumen fermentation changes which could explain the different gas composition.

5. Conclusions

Based on the changes in fermentation characteristics during 60 days ensiling of whole corn crops, the addition of mixed inoculants of 60% *L. plantarum*, 10% *P. pentosaceus*, 30% *L. buchneri*, compared with the addition of whichever single HoLAB or HeLAB inoculants, remarkably improved silage quality in terms of a sharp pH decline and greater lactate production. Meanwhile, the present study for the first time reports that mixed LAB inoculant application facilitated the release of ester-linked phenolic acids (e.g., ferulic and *p*-coumaric acids) from plant cell walls, along with enrichment of *Bacteroides*, *Bifidobacterium*, *Prevotella*, and *Bacillus* at the genus level during the ensiling process. The in vitro rumen fermentation test further demonstrated that the above mixed inoculant significantly facilitated microbial digestion of lignified fiber (e.g., ADF) in corn silage and shifted rumen fermentation towards greater propionate and lower methane production. In brief, the results obtained in the present study provided solid evidence for the role of mixed LAB application in corn silage preparation.

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