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Effects of *Lactobacillus plantarum* on Fermentation Quality and Anti-Nutritional Factors of Paper Mulberry Silage

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Abstract: There are few studies on the application of lactic acid bacteria in the reduction of anti-nutrient factors in paper mulberry silage. This study aimed to investigate the effects of different lactic acid bacteria on the fermentation quality and the amount of anti-nutritional factors in paper mulberry silage. Two strains of *Lactobacillus plantarum* (GX, isolated from paper mulberry silage; GZ, provided by Sichuan Gaofuji Biotechnology Co. Ltd.) were added as silage additives. On days 7, 15, 30 and 60 of the ensiling process, the fermentation quality, and the amount of anti-nutritional factors were measured. Compared with the control group, inoculation with *Lactobacillus plantarum* could rapidly reduce pH values, leading to lower NH_3-N/TN. Besides, it also significantly increased the lactic acid content ($p < 0.05$). The two strains of *L. plantarum* significantly reduced the content of hydrolysed tannin, condensed tannin, total tannin, oxalic acid, phytic acid and saponin ($p < 0.05$). Overall, this study found that the addition of lactic acid bacteria could significantly improve the fermentation quality of paper mulberry and reduce the amount of anti-nutrient factors ($p < 0.05$).

Keywords: *Lactobacillus plantarum*; fermentation quality; anti-nutritional factors; paper mulberry; silage

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1. Introduction

In recent years, with the rapid development of the livestock industry, the problem of insufficient sources of protein feed for animals has been increasing seriously, resulting in higher price of raw materials and competition for land use. Therefore, exploring available protein resources is essential for animal feed.

Paper mulberry (PM, *Broussonetia papyrifera* L.) is widely grown in most parts of Asia [1]. Paper mulberry has been reported to have a high level of crude protein content ranging from 18% dry matter (DM) to 22% DM, with a similar concentration as alfalfa (about 20% DM), and an acceptable content of fibre, bioactive matter and mineral substances for livestock [2]. Therefore, PM has the potential to be used as a new feed source to alleviate protein shortage and promote recent livestock recent industry development [3–5].

For various animals, there are different preferred ranges of PM supplementation. However, excessive supplementation in the feed could cause adverse reactions, and even poisoning or death to animals [6–8]. This indicates that there might be some anti-nutritional factors in PM. Anti-nutritional factors are substances that produce anti-nutritional effects on digestion, absorption, and utilization of nutrients in PM diets through synthesis during plant metabolism. Moreover, anti-nutritional factors even produce toxic effects. To improve silage quality and its tolerance threshold for livestock, it is necessary to reduce the amount of anti-nutritional factors. Ensiling had been regarded as the most effective technique to preserve nutrients and active substances of PM, and a unique way to provide nutrition-rich green feed during non-growing seasons [9,10]. In addition, ensiling has been used to reduce the concentration of anti-nutritional factors, and even toxic or harmful substances in animal feed [11,12]. Notably, lactic acid bacteria (LAB) inoculants play an important

role in improving PM silage fermentation, nutrient utilization, and reduction of anti-nutritional levels.

To date, some researchers have focused on the changes of nutritional value and active substance content after ensiling, while few have reported the reduction of anti-nutritional factors. Therefore, this study aimed to investigate the effects of LAB and ensiling time on PM silage quality and the contents of anti-nutritional factors in PM silage.

2. Materials and Methods

2.1. Experiment Materials

Broussonetia papyrifera (Zhong ke No.101) was harvested in third cut at the Zhuozhou Teaching Experimental Base (115°44′ E, 39°21′ N). Microbial additives were lactic acid bacteria GX (*Lactobacillus plantarum*, isolated from the surface of *B. papyrifera* silage) and GZ (*Lactobacillus plantarum*, purchased from Gaofuji Biotechnology Co., Ltd., Chengdu, China).

2.2. Experimental Design

The whole-plant paper mulberry was approximately 1.2 m in height. After harvest, the fresh material was immediately chopped into pieces of about 1–2 cm using a chaff cutter, and then directly ensiled by adding microbial additives. The different additives included no inoculants (CK), lactic acid bacteria GX (1×10^6 CFU·g^{-1}) or lactic acid bacteria GZ (1×10^6 CFU·g^{-1}). After the material and additives were mixed, 200 g of materials were ensiled in polyethylene bags (18 cm × 24 cm) and stored at 25–30 °C. This treatment was repeated three times. After different ensiling time (7 d, 15 d, 30 d and 60 d), we opened the bags to determinate the nutrition quality and changes in anti-nutritional factors.

2.3. Analysis of Nutritional Indexes

The pre-ensiling paper mulberry materials and silages were packed into kraft bags and dried in an air oven at 65 °C for at least 48 h to determine dry matter (DM). These dry samples were passed through a 1.5 mm sieve using a mill and stored for further chemical analysis. The content of water-soluble carbohydrates (WSC) was determined by the DNS colorimetric method [13]. The content of neutral detergent fibre (NDF) and acid detergent fibre (ADF) was analysed using an A220 Fibre Analyzer (ANKOM Technology Corp., Macedon, NY, USA) with the method of Van Soest et al. [14]. The content of total nitrogen (TN) was determined by the Kjeldahl procedure (FOSS Kjeltec TM 2300) and the CP (crude protein) was calculated by multiplying TN by 6.25 [15].

2.4. Determination of pH, Ammonia Nitrogen, and Organic Acids

Ten grams of fresh materials and each silage sample were mixed with 90 mL of sterilized distilled water (with solid-liquid ratio of 1:10), and then stored in a refrigerator at 4 °C for at least 4 h. Then, the extract materials were filtered through filter paper. The pH was immediately measured using a pH meter (PHS-3C, INESA Scientific Instrument, Shanghai, China) [16]. The content of ammonia nitrogen (NH_3-N) of each silage sample was determined according to the method of Broderick and Kang [17]. The concentrations of organic acids, including lactic acid (LA), acetic acid (AA), propionic acid (PA) and butyric acid (BA), were measured by HPLC (column: Shodex RS Pak KC-811; Showa Denko K.K., Kawasaki, Japan; detector: DAD, 210 nm, SPD-20A; Shimadzu Co., Ltd., Kyoto, Japan; eluent: 3 mmol·L^{-1} $HClO_4$,10 mL/min; temperature: 50 °C) [18].

2.5. Determination of Microbial Population

The microbial populations of the silages were determined using the plate counting method, as described by He et al., 2019 [19]. As for plate counting, 10 g of pre-ensiled materials and silage samples were immediately blended with 90 mL of sterilized water and serially diluted from 10^{-1} to 10^{-6} using sterilized water. Then, 0.02 mL of diluted suspension was spread on the corresponding agar separately after incubation at 30 °C for 2–3 days.

2.6. Determination of Anti-Nutritional Factors

The contents of total tannin, condensed tannin and hydrolysable tannin was determined by Folin-ciocalte reagent colorimetric method, slightly modified according to the method of the reference [20,21]. Determination of hydrolysable tannin: 0.1 g sample powder was added to a graduated test tube containing 2.5 mL 70% acetone. The mixtures were thoroughly shaken and treated with ultrasonic wave before collecting the supernatant. Then, 1 mL of extract suspension was pipetted to a graduated test tube containing 0.5 mL Folin reagent and 2.5 mL sodium carbonate solution. After stirring, the mixed solution was kept at 22 ± 3 °C for 40 min. The absorption at 725 nm (A) was determined using gallic acid as the reference material and a blank solution as the standard solution. The hydrolysed tannin content is the difference between total phenols and simple phenols. Determination of condensed tannin: 0.1 g sample powder was added to a graduated test tube containing 5 mL Hydrochloric acid-methanol. The mixtures were thoroughly shaken and treated with shaking for 10 min before collecting the supernatant. Then, 1 mL extract suspension was pipetted to a graduated test tube containing 5 mL vanillin solution. After stirring, the mixed solution was kept at 22 ± 3 °C for 20 min. The absorption at 495 nm (A) was determined using catechin as the reference material and a blank solution as the standard solution. The total tannin content was the sum of hydrolysed tannin and condensed tannin.

The content of oxalic acid was determined by ferric salicylate colorimetry, slightly modified according to the method of the reference [22]. First, 5 g of sample powder was added to an Erlenmeyer flask containing 50 mL distilled water. The mixtures were thoroughly shaken and heated for 30 min in a water bath before collecting the supernatant. Then, 1 mL extract suspension was pipetted to a graduated test tube containing 2 mL ferric chloride solution reagent and 20 mL buffer solution. After stirring, the mixed solution was kept at 22 ± 3 °C for 30 min. The absorption at 510 nm (A) was determined using sodium oxalate as the reference material and a blank solution as the standard solution.

The content of phytic acid was determined by ferric chloride colorimetric method, slightly modified according to the method of the reference [23]. First, 2 g of sample powder was added to an Erlenmeyer flask containing 40 mL sodium sulphate-hydrochloric acid solution. The mixtures were thoroughly shaken and treated for 30 min at a room temperature before collecting the supernatant. Then, 5 mL extract suspension was pipetted to a graduated test tube containing 5 mL 15% trichloroacetic acid reagent. After stirring and centrifugation, 5 mL dilute solution was pipetted to a graduated test tube containing 1 mL ferric chloride- sulfosalicylic acid solution. After stirring, the mixed solution was kept at 22 ± 3 °C for 20 min. The absorption at 500 nm (A) was determined using sodium phytate as the reference material and a blank solution as the standard solution.

The content of saponin was determined by vanillin-perchloric acid colorimetry, slightly modified according to the method of the reference [24]. First, 1 g of sample powder was added to a graduated test tube containing 20 mL 80% ethanol. The mixtures were thoroughly shaken and treated for 30 min in a water bath before collecting the supernatant. Then, 0.1 mL extract suspension was pipetted to a graduated test tube containing 0.1 mL 5% vanillin-acetic acid solution and 0.7 mL perchloric acid solution. After stirring, the mixed solution was kept at 67 ± 3 °C for 15 min and treated for 5 min in ice bath. Then 9.2 mL acetic acid solution was added in this tube. The absorption at 545 nm (A) was determined using oleanolic acid as the reference material and a blank solution as the standard solution.

2.7. Statistical Analysis

Two-way analysis of variance and Duncan's multiple range tests were performed to evaluate the effects of the Treatment (T), Days (D), and their interaction (T × D) on the fermentation characteristics, microbial population, and the anti-nutritional factors of silage in the general line model of IBM SPSS 21.0 software (IBM Co., Armonk, NY, USA). The level of statistical significance was set to $p < 0.05$ using SPSS. The results were expressed as mean ± standard deviation ($\overline{x} \pm s$).

3. Results

3.1. Chemical Composition, Microbial Characteristics and Anti-nutritional Factors of Fresh Paper Mulberry

The chemical compositions, microbial characteristics and anti-nutritional factors of fresh paper mulberry are shown in Table 1. The contents of CP and WSC were 131.62 g·kg^{-1} DM and 32.83 g·kg^{-1} DM, respectively. The number of lactic acid bacteria, yeast and coliform bacteria were 5.47, 2.47 and 5.44 log cfu·g^{-1} FM, respectively. The contents of anti-nutritional factors of hydrolysable tannin (HT), condensed tannin (CT), total tannin (TT), oxalic acid, phytic acid and saponin were 3.13, 13.79, 16.92, 0.89, 1.27 and 24.05 g·kg^{-1} DM, respectively.

Table 1. The characteristics of paper mulberry.

Items	Content
DM%	34.16 ± 1.95
CP g·kg^{-1} DM	131.62 ± 14.80
EE g·kg^{-1} DM	88.89 ± 2.57
WSC g·kg^{-1} DM	32.83 ± 0.72
NDF g·kg^{-1} DM	550.54 ± 14.04
ADF g·kg^{-1} DM	259.91 ± 8.56
HT g·kg^{-1} DM	3.13 ± 0.07
CT g·kg^{-1} DM	13.79 ± 1.23
TT g·kg^{-1} DM	16.92 ± 1.29
OA g·kg^{-1} DM	0.89 ± 0.09
PA g·kg^{-1} DM	1.27 ± 0.03
SA g·kg^{-1} DM	24.05 ± 0.24
LAB log cfu·g^{-1} FM	5.47 ± 0.07
YEAST log cfu·g^{-1} FM	2.47 ± 0.81
CB log cfu·g^{-1} FM	5.44 ± 0.07

DM, Dry matter; CP, crude protein; EE, ether extract; WSC, water soluble carbohydrate; NDF, neutral detergent fibre; ADF, acid detergent fibre; NH_3-N/TN, ammonia nitrogen/total nitrogen; HT, hydrolysable tannin; CT, condensed tannin; TT, total tannin; OA, oxalic acid; PA, phytic acid; SA, saponin; LAB, lactic acid bacteria; YEAST, yeast; CB, coliform bacteria.

3.2. Dynamic Changes of Fermentation Characteristics of Paper Mulberry in the Ensiling Process

The fermentation quality of paper mulberry silage is shown in Table 2. Additives had significant ($p < 0.05$) effects on DM, pH, CP, NDF, ADF, EE, LA, AA, PA, BA, and NH_3-N/TN compared with CK. Compared with CK, the treated silages exhibited better fermentation, as indicated by higher lactic acid content ($p < 0.05$) and lower pH, NH_3-N/TN ($p < 0.05$). The CP content in our group ranged from 111.61 to 112.37 g·kg^{-1} DM, and no significant difference was observed. The WSC content in CK, GX and GZ significantly decreased from 34.89 to 24.41 g·kg^{-1} DM, 44.40 to 19.91 g·kg^{-1} DM and 38.18 to 14.13 g·kg^{-1} DM. The LA content of silage significantly increased with time ($p < 0.05$), which might be due to effects of LAB.

Table 2. Fermentation characteristics of paper mulberry in the ensiling process.

Items	Treatment (T)	Days (D)				SEM	*p*-Value	T	D	T × D
		7 d	15 d	30 d	60 d					
DM	CK	34.78 ± 1.76 Aa	32.39 ± 1.04 Bb	31.61 ± 0.49 Cb	34.44 ± 0.65 Ba	0.49	0.013	*	*	*
	GX	35.00 ± 1.07 Aa	34.79 ± 0.53 Aa	36.44 ± 0.41 Ba	35.65 ± 1.20 Ba	0.29				
	GZ	35.62 ± 0.96 Aa	34.26 ± 0.95 Ab	36.96 ± 0.24 Aa	39.90 ± 3.75 Aa	0.80				
pH	CK	6.19 ± 0.04 Aa	5.92 ± 0.19 Aa	5.95 ± 0.04 Aa	4.92 ± 0.36 Ab	0.16	0.003	**	**	**
	GX	5.19 ± 0.02 Ba	4.92 ± 0.16 Bb	4.59 ± 0.05 Bc	4.35 ± 0.10 Ad	0.10				
	GZ	5.26 ± 0.11 Ba	4.80 ± 0.04 Bb	4.69 ± 0.10 Bb	4.44 ± 0.29 Ab	0.10				
CP g·kg^{-1} DM	CK	106.96 ± 0.50 Aa	102.36 ± 5.98 Aa	100.44 ± 8.77 Aa	111.61 ± 16.58 Aa	2.75	0.946	ns	*	ns
	GX	106.10 ± 8.04 Ab	98.18 ± 6.54 Ab	103.35 ± 7.84 Ab	116.00 ± 3.99 Aa	2.57				
	GZ	105.33 ± 3.76 Aa	100.07 ± 11.91 Aa	105.48 ± 3.24 Aa	112.37 ± 1.75 Aa	2.07				
NDF g·kg^{-1} DM	CK	549.91 ± 45.30 Aa	505.41 ± 50.03 Aa	499.33 ± 70.09 Aa	348.29 ± 32.51 Bb	26.20	0.002	*	**	**
	GX	445.40 ± 40.10 Ba	462.03 ± 29.25 ABa	490.59 ± 53.84 Aa	467.15 ± 16.65 Aa	10.45				
	GZ	437.98 ± 31.99 BAb	385.61 ± 51.16 Bb	468.29 ± 13.37 Aa	421.74 ± 50.72 AAb	13.33				

Table 2. *Cont.*

Items	Treatment (T)	Days (D)				SEM	*p*-Value	T	D	T × D
		7 d	15 d	30 d	60 d					
ADF g·kg^{-1} DM	CK	335.28 ± 3.07 Aa	278.56 ± 8.77 Ab	268.42 ± 36.69 Bb	209.87 ± 24.23 Bc	14.51	0.009	ns	*	**
	GX	245.54 ± 81.02 Ab	270.83 ± 26.51 AAb	348.08 ± 41.18 Aa	253.46 ± 31.04 ABAb	17.34				
	GZ	271.25 ± 31.57 AAb	252.77 ± 50.9 Ab	331.22 ± 12.91 ABa	293.42 ± 42.62 AAb	12.73				
EE g·kg^{-1} DM	CK	84.30 ± 1.17 Bb	99.31 ± 6.25 Aa	96.65 ± 6.99 AAb	98.16 ± 10.73 AAb	2.53	0.015	*	*	*
	GX	93.54 ± 6.49 Aa	91.78 ± 3.18 Aa	77.61 ± 6.45 Bb	92.48 ± 6.97 Aa	2.45				
	GZ	92.29 ± 2.03 Aa	92.82 ± 7.47 Aa	80.84 ± 3.68 Bb	87.49 ± 5.11 AAb	1.90				
WSC g·kg^{-1} DM	CK	34.89 ± 7.49 Aa	29.23 ± 1.95 Aa	25.49 ± 5.26 ABa	24.41 ± 8.29 Aa	1.97	0.117	**	*	ns
	GX	44.40 ± 3.69 Aa	24.54 ± 4.54 Abc	27.59 ± 2.14 Ab	19.91 ± 1.65 Ac	2.90				
	GZ	38.18 ± 5.32 Aa	25.31 ± 7.29 Ab	17.96 ± 3.67 Bbc	14.13 ± 3.71 Ac	3.05				
LA g·kg^{-1} DM	CK	10.00 ± 0.49 Cb	42.39 ± 10.13 Ba	46.28 ± 5.61 Aa	43.16 ± 2.91 Ba	4.49	0.009	*	**	**
	GX	21.82 ± 0.64 Bb	46.43 ± 5.15 Ba	51.68 ± 4.58 Aa	44.92 ± 2.51 Ba	3.58				
	GZ	36.96 ± 5.09 Ac	66.23 ± 4.85 Aa	49.83 ± 1.52 Ab	56.38 ± 1.69 Ab	3.78				
AA g·kg^{-1} DM	CK	26.76 ± 2.86 Bb	41.52 ± 5.77 Aa	38.29 ± 38.29 Aa	28.87 ± 7.68 Ab	2.26	0.037	*	**	**
	GX	50.68 ± 3.20 Aa	29.85 ± 2.60 Bb	27.11 ± 0.20 Bc	30.45 ± 2.02 Ab	2.89				
	GZ	25.03 ± 4.66 Ab	41.06 ± 3.87 Aa	36.22 ± 1.62 Aa	21.80 ± 4.18 Ab	2.55				
PA g·kg^{-1} DM	CK	1.74 ± 0.66 Ab	2.38 ± 0.81 Aa	1.98 ± 0.34 Bb	1.10 ± 0.28 Ac	0.20	<0.001	**	**	**
	GX	-	-	-	-	-				
	GZ	2.10 ± 0.10 Ab	1.14 ± 0.56 Bc	3.46 ± 2.48 Aa	1.51 ± 0.62 Ac	0.42				
BA g·kg^{-1} DM	CK	0.51 ± 0.01 Bc	1.55 ± 0.28 Bb	2.10 ± 0.32 Aa	0.47 ± 0.09 Bc	0.22	<0.001	**	**	**
	GX	1.78 ± 0.08 Ab	2.21 ± 0.27 Aa	0.92 ± 0.03 Bc	0.76 ± 020 Bc	0.19				
	GZ	-	-	-	-	7.92				
NH_3/TNg·kg^{-1} TN	CK	3.38 ± 0.42 Ac	3.56 ± 0.45 Ac	7.26 ± 0.47 Aa	5.61 ± 0.15 Ab	0.49	0.029	**	**	**
	GX	1.59 ± 0.28 Bc	2.39 ± 0.15 Bb	2.40 ± 0.03 Bb	3.00 ± 0.39 Ba	0.16				
	GZ	1.77 ± 0.10 Bb	2.12 ± 0.16 Bb	2.10 ± 0.28 Bb	3.65 ± 0.50 Ba	0.25				

a,b,c,d means different in the same line with different letters ($p < 0.05$); A,B,C means different in the same column with different superscripts ($p < 0.05$). ns, not detected; *, $p < 0.05$; **, $p < 0.01$. CK: no inoculants; GX: lactic acid bacteria GX as inoculants; GZ: lactic acid bacteria GZ as inoculants. CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; EE, ether extract; WSC; water soluble carbohydrates; AA, acetic acid; PA, propanoic acid; BA, butyric acid; NH_3/TN, ammonia nitrogen/total nitrogen.

3.3. Effects of Different Factors on the Microbial Characteristics of Ensiled Paper Mulberry

As presented in Table 3, the factor levels of fermentation time and *L. plantarum* treatments had significant effect on the amount of LAB ($p < 0.05$). At D60, the amount of LAB in GX was highest, with 8.58 log cfu·g^{-1} FM. In addition, the number of lactic acid bacteria was higher in GX and GZ group than in the CK ($p < 0.05$). What is more, no yeast, moulds and coliform bacteria were detected in all groups.

Table 3. Content of micro-organisms in paper mulberry in the ensiling process (log cfu·g^{-1} FM).

Items	Treatment (T)	Days (D)				SEM	*p*-Value	T	D	T × D
		7 d	15 d	30 d	60 d					
LAB	CK	5.63 ± 0.06 Ac	7.47 ± 0.07 Aa	6.69 ± 0.22 Ab	7.55 ± 0.13 Ba	0.24	<0.01	**	**	ns
	GX	6.82 ± 0.98 Ac	7.57 ± 0.43 Ab	7.31 ± 0.64 Ab	8.58 ± 0.30 Aa	0.25				
	GZ	6.95 ± 0.77 Ab	8.00 ± 0.06 Aa	6.86 ± 0.05 Ab	7.73 ± 0.60 Bab	0.19				
YEAST	CK	-	-	-	-	-	-	-	-	-
	GX	-	-	-	-	-	-	-	-	-
	GZ	-	-	-	-	-	-	-	-	-
MOULD	CK	-	-	-	-	-	-	-	-	-
	GX	-	-	-	-	-	-	-	-	-
	GZ	-	-	-	-	-	-	-	-	-
CB	CK	-	-	-	-	-	-	-	-	-
	GX	-	-	-	-	-	-	-	-	-
	GZ	-	-	-	-	-	-	-	-	-

a,b,c means different in the same line with different letters ($p < 0.05$); A,B means different in the same column with different superscripts ($p < 0.05$). ns, not detected; *, $p < 0.05$; **, $p < 0.01$. CK: no inoculants; GX: lactic acid bacteria GX as inoculants; GZ: lactic acid bacteria GZ as inoculants. LAB, lactic acid bacteria; YEAST, yeast; MOULD, mould; CB, coliform bacteria.

3.4. Effects of Different Factor Levels on Anti-Nutritional Factors of Ensiled Paper Mulberry

Anti-nutritional factors such as hydrolysable tannin, condensed tannin, total tannin, oxalic acid, phytic acid and saponin of ensiled paper mulberry are presented in Table 4. The factors of T, D, T × D in CK, GX, GZ treatments had a significant impact on anti-nutritional factors. An upward trend was observed in the content of hydrolysable tannin and condensed tannin throughout the entire ensiling process ($p < 0.05$). Total tannin were

the sum of hydrolysable tannin and condensed tannin, so the content of total tannin was increasing during ensiling ($p < 0.05$). The content of oxalic acid in ensiled paper mulberry in CK was only 0.18–0.44 g·kg^{-1} DM. Though there was a significantly difference among the three treatments, the oxalate content did not change much. The phytate content of CK was higher than GX and GZ ($p < 0.05$). The same was true for saponin. In GZ, the anti-nutritional factors, including hydrolysable tannin, condensed tannin, total tannin, phytic acid and saponin, were lower than CK and GC ($p < 0.05$) after 60 days of ensiling.

Table 4. Content of anti-nutritional factors in paper mulberry in the ensiling process (g·kg^{-1} of dry basis).

Items	Treatment (T)	Days (D)				SEM	*p*-Value	T	D	T × D
		7 d	15 d	30 d	60 d					
HT	CK	0.90 ± 0.06 Cd	1.97 ± 0.39 Bc	4.36 ± 0.60 Ab	10.53 ± 0.79 Aa	1.13	0.001	**	**	**
	GX	3.71 ± 0.43 Ac	5.75 ± 1.01 Ab	5.58 ± 0.49 Ab	8.67 ± 0.10 Ba	0.57				
	GZ	2.53 ± 0.58 Bc	4.49 ± 0.44 Ab	3.91 ± 0.98 Bb	6.65 ± 0.54 Ca	0.48				
CT	CK	22.32 ± 3.47 Ab	21.72 ± 0.89 Ab	28.54 ± 2.00 Aa	30.74 ± 0.79 Aa	1.29	0.028	**	**	*
	GX	20.21 ± 2.98 Ab	19.36 ± 1.66 Ab	23.09 ± 2.79 Bb	29.81 ± 5.17 Aa	1.49				
	GZ	23.65 ± 3.07 Aa	19.60 ± 0.95 Ab	16.77 ± 1.96 Cb	23.28 ± 1.99 Ba	3.47				
TT	CK	23.22 ± 3.43 Ac	23.69 ± 0.60 Ac	32.90 ± 2.32 Ab	41.27 ± 1.48 Aa	2.31	0.011	**	**	**
	GX	23.92 ± 3.40 Ab	25.12 ± 2.06 Ab	28.67 ± 2.62 Ab	38.48 ± 4.26 Aa	1.90				
	GZ	26.19 ± 2.73 Aa	24.09 ± 1.37 Aa	20.67 ± 2.72 Bb	29.92 ± 2.11 Ba	1.116				
OA	CK	0.23 ± 0.09 Ba	0.95 ± 0.08 Aa	0.38 ± 0.001 Ba	0.32 ± 0.001 Aa	0.08	0.003	**	**	**
	GX	0.44 ± 0.01 Ab	0.18 ± 0.03 Cc	0.65 ± 0.10 Aa	0.18 ± 0.02 Bc	0.06				
	GZ	0.43 ± 0.06 Aa	0.56 ± 0.05 Ba	0.30 ± 0.06 Cd	0.34 ± 0.01 Ac	0.03				
PA	CK	1.06 ± 0.06 Ab	1.31 ± 0.08 Aa	1.14 ± 0.01 Ab	1.10 ± 0.08 Ab	0.03	0.001	**	**	**
	GX	1.12 ± 0.02 Aa	0.98 ± 0.04 Bb	0.82 ± 0.10 Bc	0.75 ± 0.03 Bc	0.05				
	GZ	1.08 ± 0.12 Aa	0.72 ± 0.04 Cb	0.76 ± 0.06 Bb	0.65 ± 0.03 Bb	0.15				
SA	CK	12.92 ± 0.62 Cd	16.67 ± 0.08 Ab	14.54 ± 0.43 Ac	17.72 ± 0.42 Aa	0.57	0.009	**	**	**
	GX	17.29 ± 0.92 Aa	14.53 ± 0.98 Bb	12.52 ± 0.64 Bc	16.75 ± 0.43 Aa	0.60				
	GZ	14.59 ± 0.83 Bb	16.48 ± 0.55 Aa	12.20 ± 1.05 Bc	14.09 ± 0.64 Bb	0.50				

a,b,c,d means different in the same line with different letters ($p < 0.05$); A,B,C means different in the same column with different superscripts differ ($p < 0.05$). ns, not detected; *, $p < 0.05$; **, $p < 0.01$. CK: no inoculants; GX: lactic acid bacteria GX as inoculants; GZ: lactic acid bacteria GX as inoculants. HT, hydrolysable tannin; CT, condensed tannin; TT, total tannin; OA, oxalic acid; PA, phytic acid; SA, saponin.

4. Discussion

Though different studies were conducted with same species of PM, there was a distinct difference in nutrient composition compared with previous studies. In this experiment, the CP content of fresh PM (131.62 g·kg^{-1} DM) was comparable to the data reported by Zhang (179.5 g·kg^{-1} DM) [25], but much lower than that reported by Du (299.0 g·kg^{-1} DM) [26]. What is more, the contents of ADF and NDF were 259.91 g·kg^{-1} DM and 550.54 g·kg^{-1} DM, respectively, which was different from the results reported by Zhang (ADF and NDF were 210.0 g·kg^{-1} DM and 300.0 g·kg^{-1} DM) [25]. Researchers found that these differences in nutrient composition might be caused by different locations, cultivate management, harvest time and treatments.

Researchers thought that the competition and collaboration between harmful microorganisms and LAB influenced the PM silage quality [27]. In fresh PM, the number of LAB (5.47 log cfu·g^{-1} FM) reached the standard for making qualified silage [28]. However, undesirable microorganisms containing yeast (2.47 log cfu·g^{-1} FM) and *E. coli* (5.44 log cfu·g^{-1} FM) were found, which were detrimental to PM silage. At the silage seeding stage, spoilage microorganisms multiplied rapidly, thus inhibiting LAB from becoming the dominant bacteria. This may lead to the failure of PM forming silage.

The number of epiphytic LAB is usually much lower than the undesirable microorganism and leads to the low efficient utilization of WSC. The WSC is the mainly energy for LAB growth, so its content plays a critical role in well-preserved silage. More importantly, inadequate epiphytic LAB number and WSC content could accelerate clostridial activity and produce high concentrations of butyric acid and ammonia-N, thus leading to poor silage [29]. To monitor the quality of PM silage, the pH value is considered one

of the major parameters. Researchers have reported that pH below 4.5, could inhibit the growth of spoilage bacteria [30]. Moreover, a pH of 4.2 is considered as a key marker for well-conserved silage [31]. In this research, the addition of LAB could reduce the pH rapidly. This indicated that the addition of LAB promoted the production of lactic acid to lower the pH of PM silage. The LAB produced a large amount of lactic acid, which rapidly lowered the pH. High lactic acid and low pH inhibited spoilage microorganisms, reduced the consumption by harmful bacteria and better retained the nutrients in PM silage. During the final 60 days, the pH of both treatments was still higher than 4.5 despite the addition of LAB. Therefore, adding exogenous *Lactobacillus plantarum* could improve the relative abundance of LAB, and ensure the production of successful PM silage. Besides, the fresh PM was harvested by hand with a sickle. Compared with mechanical harvesting, the degree of harvesting by hand was not conductive to the utilization of WSC in raw PM by bacteria [32].

The crude protein amount [33] is one of the important indicators to evaluate the nutritional quality of silage. The ammonia nitrogen in silage, which cannot be used by animals, is an indicator mirroring the degree of protein reduction [34]. Lower ammonia nitrogen values indicates less reduction of CP; small values for peptide and free amino acids, also indicate higher protein retention rate and better silage quality. In this study, the addition of LAB (GX and GZ) increased CP content and reduced NH_3-N/TN ($p < 0.05$) levels. The explanation was that LAB could inhibit the activity of related microorganisms and inhibit the production of enzymes that caused protein degradation.

The anti-nutritional factors are also important in the utilization of PM, but data for anti-nutritional factors in PM are less well reported. Oxalic and phytic acids form complexes with minerals in diets, resulting in mineral-related deficiency in animals. What is more, oxalate and phytate also show negative impact on protein and lipid utilization [35]. Values of 5 g·kg^{-1} and 20 g·kg^{-1} oxalate in diet cause toxicity to monogastric and ruminant animals, respectively [36]. The content of phytic acid, an anti-nutritional factor in fava beans, ranges from 19.65 g·kg^{-1} DM to 22.85 g·kg^{-1} DM. The content of phytic acid in oat, wheat, sorghum, and soybean was 8.8, 10.3, 10.8 and 14.0 g·kg^{-1} DM, respectively [37], which is higher than of flesh PM phytate (1.27 g·kg^{-1} DM). But in this study, the content of oxalic acid and phytic acid in PM was 0.89 g·kg^{-1} and 1.27 g·kg^{-1} DM, respectively, indicating that oxalate and phytate in PM showed no negative impact. Researchers have reported that diets with more than 5 g total tannin/kg would reduce fodder intake in chickens and increase mortality in rats [36]. In addition, young animals were more sensitive to dietary TT than adult animals. Some studies have observed that pigs are sensitive to hydrolysable tannin ranging 0.125 g HT/kg to 1.0 g HT/kg [38]. High tannin content (more than 55 g·kg^{-1} DM) of sorghum is a terrible characteristic for animals [39]. The content of tannin, the main anti-nutritional factor in leaves of *Moringa oleifera*, is 8.39 g·kg^{-1} DM [40]. Compared with this, tannin is the main anti-nutritional factor in PM. While using PM as unconventional feed, we should pay more attention to the tannin content. Saponins affect animal performance, and poultry are more sensitive to saponins. Chicks show distinct growth depression with a diet of 3 g saponins/kg [36]. It was found that the saponins content of alfalfa ranged from 0.7 to 3.3 g·kg^{-1} DM [41]. The saponins content of fresh PM is 24.05 g·kg^{-1} DM, higher than that of alfalfa. In addition, Suárez-Estrella has found that the bitterness of plants was associated with saponins higher than 1.1 g·kg^{-1} DM [42]. Saponins are one of the main anti-nutritional factors in PM. In this study, hydrolysable tannin, total tannin and saponins in PM are likely to constitute a hindrance to nutrients digestion and cause a negative impact on animals.

Ensiling using lactic acid bacteria (LAB) can improve the quality of silage and reduce anti-nutritional factors [43]. In this study, hydrolysable tannin, condensed tannin, and total tannins showed an upward trend with the time. Tannin was combined with peptides to form tannin-peptides complexes which could protected protein from degradation [44]. With higher content of tannin, the PM protein could be better preserved. Li [45] also found that ensiling increased condensed and hydrolysed tannin significantly in paper mulberry.

Abbasi found that the content of tannin in *Amaranthus hypochondriaus* silage decreased from 7.82 g·kg^{-1} DM to 5.77 g·kg^{-1} DM, with a degradation rate of 26.21% [46]. The decrease in condensed tannin level from 2.84% to 2.34% was also found in *Zea mays* [47]. The decrease in tannin content was realized through acylhydrolase tannin activity. These differences may result from the fact that the tannin enzyme activity of GX and GZ was not identified. The trend of tannin in silage has not been determined yet. Physical composition, chemical properties and nutrient composition of forage grass are closely related to the species of forage grass and the environmental conditions during silage production. With the decreased pH value during the ensiling process, condensed tannin will be polymerized at the interconnecting part of C-C bond to form the flavan-3-ol, whose derivative is catechin. Catechin was selected as the standard substance to determine the content of condensed tannin in our study. Total tannin is a summary of hydrolysed and condensed tannin. With the increase of condensed tannin and hydrolysed tannin, total tannin increased [48].

The selected strains of GX and GZ showed an effective reduction of phytic acid and saponins. This observed reduction maybe be caused by phytases and glycosidase, which were reduced by LAB, while LAB could hydrolyse phytic acid to inositol and saponin to glycosides, respectively [49,50], during the fermentation process compared to the GX. This can be explained by that GZ produced more relative enzymes to reduce phytic acid and saponins. The lactic acid bacteria can produce phytase enzyme, which can degrade phytate. The reduction in phytate can be attributed to production of phytase enzyme during the fermentation process. Phytic acid degradation is pH dependent and the optimal pH for most phytases ranges between 4.0 and 6.0. Possibly, a reduction in the pH could have activated phytase to reduce the levels of phytic acid [51,52]. Researchers have found that the total saponin content decreased during ensiling, and the saponin content was positively correlated with pH (0.555) and the acetate (0.243) and ammonia N (0.271) contents [53].

5. Conclusions

Over 60 days, the data from our study showed that adding *Lactobacillus plantarum* (GX and GZ) for PM silage can significantly improve the fermentation quality of PM. In addition, the addition of GX and GZ also reduced the amount of the anti-nutritional factors, including phytic acid and saponin in PM ($p < 0.05$). This study provided new insights into the reduction of anti-nutritional factors by the addition of *Lactobacillus plantarum* to PM silage. A downward trend was observed in the content of phytic acid and saponin throughout the entire ensiling process ($p < 0.05$). The same was true for pH.

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