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Full Length Article

Effect of *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Lactobacillus buchneri* at Low Doses on the Fermentation, Aerobic Stability and Ruminal Digestibility of Corn Silage

Fang Miao[1†], Fanfan Zhang[1†], Xuzhe Wang[1], Shuan Jia[1,2], Weihua Lu[1] and Chunhui Ma[1*]
[1]*College of Animal Science and Technology, Shihezi University, Xinjiang, 832000, China*
[2]*Institute of Animal Health Supervision, Urumqi, Xinjiang, 830011, China*
[*]For correspondence: zhangfanfan@shzu.edu.cn; chunhuima@126.com
[†]These authors contributed equally in this work

Abstract

The effect of the combination of two kinds of homofermentative lactic acid bacteria (LAB) *viz*. *Lactobacillus plantarum* and *Pediococcus acidilactici*, alone or in combination with *Lactobacillus buchneri* on the fermentation characteristics, aerobic stability and ruminal digestibility of corn silage was investigated under laboratory conditions. The following fermentation systems were established: (1) the control was untreated silage with no inoculant applied; (2) LP treatment was a 1:1 mix of *L. plantarum* and *P. acidilactici* at 1×10^5 cfu/g of wet silage; (3) LB treatment was *L. buchneri* at 1×10^5 cfu/g; and (4) LPLB treatment was the three kinds of LAB combined 1:1:1, each at 1×10^5 cfu/g. A total of 84 anaerobic jars were prepared for the corn silage systems. Three jars per treatment were sampled on day 2, 4, 8, 15, 35, 45, and 60. After 60 days of fermentation, the rumen digestibility of the silage was measured by an in-situ procedure, and the silages were subjected to an aerobic stability test that lasted for 5 days. Compared with the other treatments, the LPLB treatment increased the number of LAB and most effectively improved the lactic acid and crude protein contents during the fermentation period. None of the inoculation treatments significantly reduced the pH of the fermentation system. However, inoculation with *L. buchneri* maintained the fermentation system at a low acidity from 35 to 60 days. The most marked degradation of neutral detergent fibre and acid detergent fibre occurred in the LB and LP treatments. Additionally, these two treatments also significantly increased the effective degradation of dry matter and neutral detergent fibre. The LB treatment improved the effective degradation of organic matter in the rumen of sheep and improved the dry matter on the 60th day of fermentation. None of the inoculation treatments significantly inhibited the growth of moulds during the fermentation process. Moreover, after 5 days with the silage jar open, none of the treatments significantly increased the number of LAB. However, the production of CO_2 was markedly decreased with all the inoculation treatments. Compared with the control condition, the LB and LPLB treatments improved the aerobic stability time. None of the inoculation treatments significantly impacted the contents of acetic acid, water-soluble carbohydrates or starch. © 2019 Friends Science Publishers

Keywords: Aerobic stability; Corn silage; Digestibility of rumen; Fermentation; Lactic acid bacteria

Introduction

Corn silage plays an important role in the ruminant breeding industry because it markedly preserves the nutritional value of green feed. Silage depends on lactic acid produced by the anaerobic fermentation of lactic acid bacteria (LAB), which reduces pH, leaf respiration and enzyme activity to subsequently inhibit the growth of mould, *Clostridium*, yeast and other adverse microorganisms to preserve feed (Keller *et al.*, 2013; Kharazian *et al.*, 2017). Therefore, one of the most important aspects of the silage fermentation process is the inoculation of LAB into silage to promote fermentation and improve fermentation quality (Adesogan and Salawu, 2010; Keller *et al.*, 2013). The LAB in silage is mainly divided into homo- and heterofermentative LAB according to different fermentation products: homofermentative LAB include *Lactobacillus plantarum*, *Streptococcus faecium* and *Pediococcus pentosaceus*, while heterofermentative LAB include *Lactobacillus brevis*, *Lactobacillus buchneri*, etc (Mcdonald *et al.*, 1991). The lactic acid content of corn silage inoculated with *L. plantarum* was increased compared with that of uninoculated corn silage (Liu *et al.*, 2016), and the inoculation of other *Lactobacillus* spp. or *Pediococcus* spp. result in the same effect (Dunière *et al.*, 2013). Although homofermentative LAB produced more lactic acid, it consumed less energy and rapidly reduced the pH of corn silage. Moreover, it effectively reduced the content of ethanol and ammonium nitrogen and increased the ratio of lactic acid

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to acetic acid. However, it produced low levels of volatile fatty acids (mainly acetic acid) by fermentation, which could not effectively inhibit the growth of moulds and other spoilage strains, resulting in the spoilage of silage (Filya *et al*., 2007; Lindseyj and Limin, 2010). *L. buchneri* produced antibacterial by-products such as acetic acid and bacteriocin, which reduced aerobic deterioration and improved silage quality (Rabelo *et al*., 2016). Therefore, the application of appropriate LAB inoculates could ensure silage quality, prevent silage deterioration, and improve economic efficiency (Huisden *et al*., 2009). During fermentation, the inoculation of the combination of *P. pentosaceus* and *L. buchneri* resulted in high acetic acid and ethanol contents and low water-soluble carbohydrate contents but could not continuously improve the aerobic stability of silage (Kleinschmit and Kung, 2006). However, the combined inoculation of LAB of the same fermentation type, *L. plantarum* and *L. buchneri*, effectively reduced the pH, protein degradation rate, and fermentation loss in the fermentation system, as well as continuously improved the aerobic stability of silage (Filya, 2003a; Reich and Kung, 2010). In addition, a large number of studies have also confirmed that the addition of other types of homo- and heterofermentative LAB has the potential to improve the fermentation quality and aerobic stability of corn silage (Oliveira *et al*., 2017; Fanfan *et al*., 2018). However, some previous studies showed that compared with *L. buchneri* alone, inoculation with *L. plantarum* and *L. buchneri* in corn silage did not significantly improve aerobic stability (Huisden *et al.*, 2009). At the same time, many studies have also shown that the combination of homo- and heterofermentative LAB did not significantly improve the fermentation quality of corn silage (Ranjit and Kung, 2000; Sadeghi *et al*., 2012; Yitbarek and Tamir, 2014). Therefore, controlling the type, proportion and effective dose of LAB might be key influencing factors. It is generally believed that the inoculation of at least 1×10^5 colony-forming units (cfu)/g LAB can inhibit the harmful epiphytic bacteria of silage from becoming the dominant populations (Li *et al*., 2016; Abdul *et al*., 2017; Oliveira *et al*., 2017). Thus, the purpose of this study was to explore the fermentation, aerobic stability, and ruminal digestibility of corn silage with a combination of *L. plantarum*, *P. acidilactici*, and *L. buchneri* at the minimum inoculation level (1×10^5 cfu/g). The aim of this study was to clarify whether the addition of different types of fermentative LAB at low doses can improve the fermentation quality, aerobic stability and rumen digestibility of corn silage and reduce the number of harmful microorganisms.

Materials and Methods

Silage Preparation and Sampling

The silage material used *Zea mays* 'Xingsiyu NO. 10'. The test site was located at the pasture station of Shihezi University (44° 20′ N, 88° 30′ E, 420 m above sea level). The growth period of silage corn was from April 10 to August 20, 2017 for a total of 112 days. The harvest time of silage corn was the period when the milk line exceeded 2/3. The whole plant was harvested and chopped into 1-2 cm pieces. Silage was immediately placed in a small silage jar (10-L anaerobic PVC jar). The following LAB were inoculated: two kinds of homofermentative LAB from the agricultural culture collection of china (ACCC), *L. plantarum* (ACCC 11016) and *P. acidilactici* (ACCC 05481); one heterofermentative LAB from the chinese center of industrial culture collection (CICC), *L. buchneri* (CICC 20293). The following four inoculation treatments were administered: (1) untreated silage with no inoculant applied (control); (2) 1:1 *L. plantarum* and *P. acidilactici* at 1×10^5 cfu/g of wet silage (LP treatment); (3) *L. buchneri* at 1×10^5 cfu/g (LB treatment); (4) three kinds of LAB combined at a 1:1:1 ratio at 1×10^5 cfu/g (LPLB treatment). Each LAB strain was enriched with MRS liquid medium. After the number of LAB was determined by counting the plate, the bacteria were evenly sprayed on the corn silage surface for storage. A total of 84 jars were prepared with silage, three jars per treatment (density of 300 kg/m^3) and samples were randomly opened during the experimental period (2, 4, 8, 15, 35, 45, 60 d), take three samples per jar for measuring chemical and microbial.

Chemical and Microbial Analyses

The LAB were incubated on MRS agar plates at 37°C for 2 days under anaerobic conditions, and the number of cfu was counted. Moulds were cultured by inoculation on malt extract agar acidified with lactic acid to a pH of 4.0. The plates were incubated at 30°C for 3 days, and the number of cfu was counted on the third day.

The dry matter contents of corn silages were determined after drying at 60°C in a forced-air oven for 48 h. Crude protein was analysed using the Kjeldahl method (AOAC, 1990). The water-soluble carbohydrates were quantified by the anthracenone sulphuric acid method (Dubois *et al*., 1956). Neutral detergent fibre and acid detergent fibre were estimated by the method of Van Soest *et al*. (1991). Starch was analysed using the hydrolysis methods described by Kartchner and Theurer (1981). The pH was measured using a pH metre (model S-35; Rex Electric Chemical, China). The lactic acid, acetic acid and butyric acid were determined in aqueous extracts by means of derivatization with gas-liquid chromatography adapted from Filya (2003a). The ammonium nitrogen was analysed by the phenol-hypochlorite procedure described by Weatherburn (1967).

Aerobic Stability Test

At the end of the fermentation period (days 60), the corn silage samples were subjected to a 5-day aerobic stability test at 23±1°C in the laboratory with the CO_2 determination method described by Ashbell *et al.* (1991). In this method,

the production of CO_2 production, change in pH, and number of LAB, moulds serve as spoilage indicators. Aerobic stability was defined as the time when the internal temperature of corn silage exceeded the environment temperature of 2°C (Filya, 2003a; Huisden *et al*., 2009; Adesogan and Salawu, 2010) as determined by a multipoint real-time temperature recorder (model I500-E3TW, Yuhuan Zhituo Instrument Technology Co. Ltd., China).

Rumen Digestibility

The rumen digestibility of the silage was measured by the in situ procedure of Mehrez and Ørskov (1977). Ten Kazakh Sheep with good growth (body weights approximately 52.0±2.50 kg) were used, and a permanent rumen fistula was installed. The sheep were pre-fed for one month before the start of the experiment, during which the adaptation of the sheep after the installation of the fistula was checked. After the end of the one-month acclimation period, three sheep were randomly selected for testing. The corn silage feed was pre-fed for one week before the test. Each sheep was fed 200 g of concentrate (Tiankang Animal Husbandry Biotechnology Co. Ltd., China) and 1.8 kg corn silage per day and was allowed to freely drink. After the start of the test, each treated sample was dried (65°C) again to a constant mass, sieved (0.425 mm), and placed in a nylon bag (size 9 × 10 cm, aperture 10 to 40 um). Each bag was accurately filled with 3 g of sample (± 0.01 g). The end of the nylon wire tie was tied to the chain and the other end was placed in the rumen after filling. At 8:00 in the morning, a total of 5 time points (12, 24, 36, 48, 72 h) were set. At each time point, the nylon bag was removed and quickly washed to measure dry matter, neutral detergent fibre, acid detergent fibre and organic matter content. Inorganic matter was determined by ashing after 3 h at 550°C and converted from inorganic matter into organic matter content. The formulas (I-III) were used to calculate the degradation rate of the corresponding indicators, namely, dry matter digestibility, neutral detergent fibre digestibility, acid detergent fibre digestibility and organic matter digestibility.

Statistical Analysis

The rumen degradation rate of corn silage was calculated with formula I, and the characteristic degradation parameters were calculated with formulas II and III (Mehrez and Ørskov, 1977; McDonald *et al*., 1991): (I) $D_x=[(M_A-M_B)/M_A]\times100\%$; (II) $D_P=A+B(1-e^{-Ct})$; and (III) $ED=A+(B\times C)/(k+C)$, where D_x is the rumen degradation rate (%) of the silage corn component to be tested, M_A is the content of the silage corn component to be tested (g), M_B is the content of the component in the rumen residue (g), D_P is the degradation efficiency of the silage corn component in the rumen for a t time, A is the rapid degradation percentage, B is the slow degradation percentage (%), $A+B$ is the potential degradation percentage, C is the degradation rate of the slowly degraded part (%/h), ED is the effective degradation rate of the component in the rumen, k is the rumen outflow rate constant (%/h) of the silage, and the k value was 4.5%/h. The partial least squares calculation was performed by using MATLAB software (v 2014).

Any significant differences between the means were identified from the *P*-values of the ANOVA, and the effects were considered significant at $P < 0.05$. When the calculated values of *F* were significant, Duncan's multiple range test ($P < 0.05$) was used to interpret any significant differences among the mean values. Additionally, two-way ANOVA was used to calculate the significant influence ($P < 0.05$ and $P < 0.01$), and Duncan's multiple range test was used to calculate the interaction of days and treatments. All of the above statistical analyses were performed using Version 13 of SPSS for Windows (SPSS Inc., Chicago, IL).

Result

Microbial, Fermentation and Nutritional Characteristics

The results showed that the initial (0 d) number of LAB in the fermentation system was 8.44 log cfu/g, which decreased during the fermentation process *i.e*., over 60 days (Table 1). The LP and LPLB treatments had significantly higher numbers of LAB than the control treatment ($P<0.05$) on the 8th and 15th days. On the 35th day, the LPLB treatment had a significantly higher number of LAB than the control treatment ($P<0.05$). However, it had no significant effect at the other fermentation time points ($P>0.05$). The number of initial mould in the fermentation was 5.77 log cfu/g, which decreased during the fermentation period. The number of moulds was significantly lower in only the LP treatment group than in the control group ($P<0.05$) on the second day. However, when the number of mould was <2.00 log cfu/g, this number could not be effectively determined.

The initial pH of the fermentation was 5.33, which decreased rapidly after the start of fermentation. On the second, 4th and 15th days, the pH of the control treatment was significantly lower than that of the LP and LPLB treatments ($P<0.05$). The pH of all the inoculation treatment groups was higher than that of the control group ($P<0.05$) on the 8th and 35th days. On the 45th and 60th days, compared with the control condition, the LB treatment had a decreased pH, and the LPLB treatment had an increased pH ($P<0.05$). The initial lactic acid content of the fermentation system was 0.61%. The lactic acid content accumulated with fermentation time, reaching its peak on the 8th day and then declining. The lactic acid content of all inoculation treatments was higher than that of the control condition ($P<0.05$) on the second and 35th days. Moreover, the lactic acid content of the LB and LPLB treatments were significantly higher than that of the control condition ($P<0.05$) on the 4th day. On the 60th day, only the LPLB treatment had an lactic acid content that was significantly higher than the lactic acid content of the control condition ($P<0.05$).

Table 1: The effect of different treatments on the microbial composition, fermentation characteristics and nutritional quality of corn silage over 60 days

Day	Treatments	Log cfu/g wet silage		pH	% Wet silage basis			Dry matter	% dry matter basis				
		Lactic acid bacteria	Mould		Lactic acid	Acetic acid	Ammonium nitrogen		Crude protein	water-soluble carbohydrate	Starch	Nneutral detergent fibre	Acid detergent fibre
0 d (Ensiling)		8.44	5.77	5.33	0.61	0.65	0.019	42.27	7.31	22.01	27.73	50.09	29.14
2 d	Control	8.93	4.95 a	4.47 b	3.84 d	3.00	0.030 ab	35.35 b	7.21 b	21.26 a	26.89 a	49.32	28.20
	LP	9.00	4.19 b	4.79 a	4.16 c	2.88	0.023 bc	38.37 a	7.25 b	17.52 b	23.18 b	49.08	28.25
	LB	8.86	4.37 ab	4.47 b	4.65 b	2.18	0.040 a	35.69 b	7.15 b	18.02 b	23.95 b	49.93	28.51
	LPLB	8.85	4.89 a	4.75 a	5.38 a	4.32	0.015 c	36.08 b	7.48 a	17.47 b	23.85 b	50.08	28.82
	SEM	0.045	0.098	0.009	0.040	0.337	0.002	0.354	0.019	0.164	0.225	0.369	0.449
4 d	Control	8.68	<2.00	4.26 c	4.72 b	3.00	0.039 ab	33.56 b	6.98 b	18.86 a	24.60 a	49.85	27.85
	LP	8.92	<2.00	4.48 b	5.42 b	3.08	0.035 ab	36.09 a	6.99 b	16.47 c	21.05 c	49.63	27.99
	LB	8.78	2.49	4.31 c	7.29 a	3.21	0.028 b	35.01 ab	6.95 b	17.52 b	23.69 b	49.48	27.45
	LPLB	8.91	3.73	4.64 a	7.05 a	2.83	0.046 a	35.66 ab	7.27 a	17.04 b	21.78 c	49.67	27.65
	SEM	0.043	0.250	0.011	0.256	0.117	0.002	0.327	0.040	0.075	0.103	0.393	0.354
8 d	Control	8.26 b	<2.00	3.79 d	6.00	3.36 a	0.042 b	35.58	6.92 ab	15.46 a	22.20 a	48.65	27.28
	LP	8.77 a	<2.00	4.21 b	6.97	0.77 b	0.064 b	37.39	6.79 c	12.71 c	18.55 c	48.34	27.57
	LB	8.30 b	<2.00	3.92 c	5.59	1.52 b	0.058 b	35.59	6.87 bc	13.96 b	20.96 b	48.67	27.91
	LPLB	8.99 a	<2.00	4.39 a	5.69	2.23 b	0.101 a	35.39	7.01 a	13.12 c	18.04 c	48.62	27.27
	SEM	0.051	-	0.016	0.288	0.240	0.004	0.443	0.016	0.090	0.383	0.370	0.260
15 d	Control	7.68 b	3.40	4.12 c	2.43	3.22	0.060 b	33.95 b	7.02 b	11.77 a	19.52 a	48.34 a	27.11 a
	LP	8.36 a	4.08	4.33 b	2.70	3.02	0.083 b	36.32 a	6.89 c	9.48 c	17.18 c	46.60 b	25.79 b
	LB	7.57 b	4.00	4.08 c	2.78	2.73	0.076 b	36.25 a	7.10 ab	10.44 b	18.76 b	46.23 b	25.58 b
	LPLB	8.50 a	4.22	4.47 a	2.67	3.37	0.159 a	35.14 ab	7.14 a	10.26 b	16.28 d	48.50 a	26.59 a
	SEM	0.056	0.104	0.015	0.061	0.157	0.006	0.263	0.015	0.053	0.220	0.151	0.105
35 d	Control	7.73 bc	3.70 b	4.02 d	1.42 c	1.29	0.044 b	34.70	6.79 b	8.75 a	18.28 a	47.81 a	27.05 a
	LP	8.16 b	4.18 a	4.32 b	1.68 b	1.17	0.089 a	35.65	7.04 a	7.64 c	16.65 b	46.16 b	25.29 b
	LB	7.46 c	3.40 b	4.09 c	1.77 b	2.41	0.057 b	33.82	7.05 a	8.21 b	18.35 a	46.12 b	25.13 b
	LPLB	8.59 a	4.41 a	4.42 a	2.03 a	1.99	0.107 a	34.94	7.11 a	7.98 b	16.86 b	47.58 a	25.95 b
	SEM	0.097	0.054	0.004	0.034	0.228	0.005	0.522	0.018	0.091	0.291	0.526	0.724
45 d	Control	7.78 a	3.60 c	4.29 b	2.70 ab	1.45	0.128 b	34.69 ab	6.90 b	7.52 a	17.53 a	46.77	26.85 a
	LP	7.87 a	4.08 b	4.26 b	2.75 ab	0.84	0.112 b	36.35 a	6.97 ab	6.66 c	16.09 b	46.82	25.84 b
	LB	7.07 b	3.39 bc	4.12 c	2.57 b	2.39	0.107 b	33.86 b	6.98 ab	6.91 b	17.85 a	46.17	25.48 b
	LPLB	7.95 a	4.87 a	4.40 a	3.05 a	2.14	0.183 a	35.72 a	7.02 a	6.40 d	16.32 b	46.68	25.60 b
	SEM	0.109	0.088	0.013	0.071	0.437	0.004	0.264	0.011	0.037	0.242	0.342	0.476
60 d	Control	6.89	3.31 c	4.09 b	1.97 b	1.39 ab	0.075 c	33.31 c	7.05	6.24 a	17.08 a	46.29 a	26.53 a
	LP	7.24	3.94 ab	4.10 b	2.06 ab	0.30 b	0.083 bc	36.17 b	7.01	5.45 c	15.55 b	45.07 b	25.56 b
	LB	7.37	3.16 bc	3.98 c	2.26 ab	2.29 a	0.119 ab	37.34 a	6.93	5.92 b	17.30 a	45.22 b	24.29 c
	LPLB	7.67	4.14 a	4.25 a	2.35 a	2.18 a	0.134 a	35.66 b	7.01	5.65 c	15.78 b	46.54 a	25.24 b
	SEM	0.155	0.111	0.012	0.054	0.213	0.006	0.256	0.018	0.041	0.236	0.186	0.122
P	M	**	**	**	**	*	**	**	**	**	**	*	*
value	D	**	**	**	**	**	**	**	**	**	**	NS	NS
	M×D	*	**	**	**	NS	**	NS	**	**	**	NS	NS

Control = untreated corn silage with no inoculant applied, LP= 1:1 *Lactobacillus plantarum* and *Pediococcus acidilactici* at 1×10^5 cfu/g, LB= *Lactobacillus buchneri* at 1×10^5 cfu/g, LPLB= 1:1:1 *Lactobacillus plantarum, P. acidilactici* and *L. buchneri* at 1×10^5 cfu/g. M= treatment, D= day, M×D= treatment × day. SEM= standard error of mean. $^{a\text{-}c}$Means within the same day and column followed by different letters are significantly different at the 5% level according to the Duncan's multiple range test. *= P<0.05, **= P<0.01, NS= not significant. butyric acid not detected. n=9

The initial acetic acid content of the fermentation system was 0.65%, and it increased to approximately 2-3% after 2 days of fermentation. Afterwards, the acetic acid content remained basically unchanged throughout the fermentation process. The initial ammonium nitrogen content of the fermentation system was 0.019%, which gradually increased with prolonged fermentation time. The ammonium nitrogen content of the LPLB treatment was significantly lower than that of the control condition (P<0.05) on the second day. However, the ammonium nitrogen content of the LPLB treatment was higher than that of the control condition (P<0.05) from day 8 to day 60 (Table 1).

The initial dry matter content of the fermentation system was 42.27%, and the dry matter content gradually decreased with the decomposition of chemical substances. The dry matter content of the LP treatment was significantly higher than the control condition (P<0.05) on the second and 4th days. The dry matter content of the LP and LB treatments were significantly increased compared with that of the control (P<0.05) on the 15th day. Compared to the control condition, all inoculation treatments resulted in increased dry matter content (P<0.05) on the 60th day. The initial crude protein content of the fermentation system was 7.31%, and there were few changes in the crude protein content during the fermentation period. On the second and 4th days, the crude protein content of the LPLB treatment was the highest of all the experimental conditions (P<0.05). The crude protein content of the LP treatment was significantly lower than that of the control condition (P<0.05) on the 8th and 15th days, while the crude protein content of the LPLB treatment was

significantly higher than that of the control condition (P<0.05) on the 15th and 45th days. On the 35th day, the crude protein contents of all inoculation treatments were higher than that of the control condition (P<0.05). The initial water-soluble carbohydrates content of the fermentation system was 22.01%; however water-soluble carbohydrates are utilized by microorganisms during the fermentation process and thus rapidly decreased in the fermentation system. On the second day, the water-soluble carbohydrates contents of all inoculation treatments were significantly lower than that of the control condition (P<0.05). The water-soluble carbohydrates content of the LP treatment was the lowest (P<0.05) on the 4th day. The water-soluble carbohydrates contents of LP and LPLB treatments were the lowest (P<0.05) on the 8th and 60th days. The water-soluble carbohydrates content of the LP treatment was the lowest (P<0.05) from day 15 to day 35. On the 45th day, the water-soluble carbohydrates content of the LPLB treatment was the lowest (P<0.05). The initial starch content of the fermentation system was 27.73%, and the variation of the starch content during fermentation was similar to that of the water-soluble carbohydrates content. On the second, 8th and 15th days, the change in the starch content was the same as that of the water-soluble carbohydrates content. On the 4th, 35th, 45th and 60th days, the starch contents of both the LP and LPLB treatments were significantly lower than those of the other treatments (P<0.05). The initial neutral detergent fibre content of the fermentation system was 50.09%, and the neutral detergent fibre content showed little change during the fermentation period. The neutral detergent fibre contents of the LP and LB treatments were significantly lower than that of the other treatments (P<0.05) on the 15th, 35th and 60th days. The initial ADF content of the fermentation system was 29.14%, which was similar to that of the neutral detergent fibre content during the fermentation period. On the 15th day, the ADF content was the lowest in the LP and LB treatments (P<0.05), and the acid detergent fibre contents of all inoculation treatments were lower than that of the control conditions (P<0.05) beginning on day 35. On the 60th day, the acid detergent fibre content of the LB treatment was the lowest (P<0.05). The neutral detergent fibre and acid detergent fibre contents were not significantly different among the experimental conditions during the rest of the treatment period (P>0.05) (Table 1).

Two-way analysis of variance showed that treatment (M) had significant effects on the acetic acid, neutral detergent fibre and acid detergent fibre contents (P<0.05) and extremely significant effects on the other indexes (P<0.01). The fermentation day (D) had no significant effect on the contents of neutral detergent fibre and acid detergent fibre (P>0.05), while the other indexes were extremely significantly impacted by fermentation day (P<0.01). The interaction between treatment and fermentation day (M×D) had a significant effect on the number of LAB (P<0.05), no significant effects on the acetic acid, dry matter, neutral detergent fibre and acid detergent fibre contents (P>0.05), and extremely significant effects on the remaining indexes (P<0.01) (Table 1).

Aerobic Stability of Silage Corn

The results showed that the pH of the LP and LPLB treatments was significantly higher than that of the control condition (P<0.01) and that the pH value of the LPLB treatment was the highest (4.21) (Table 2). The CO_2 gas production of the control condition was the highest of all the experimental groups (5.22 g/kg) and significantly higher than that of the inoculation treatments. The lowest number of moulds was observed in the control condition (3.08 log cfu/g); the number of moulds in the control condition was extremely significantly lower than that in the inoculation treatments (P<0.01). The aerobic stability times of the LB (195.58 h) and LPLB (196.21) treatments were significantly longer than those of the LP treatment and control condition (P<0.05). The difference in the number of LAB was not significant among the experimental conditions (P>0.05).

Degradation Characteristics of Silage Corn in the Rumen

After analysing the dry matter, neutral detergent fibre, acid detergent fibre and organic matter digestibility of corn silage in the rumen, the results showed that the organic matter digestibility of the LB treatment was significantly higher than that of the other treatments (P<0.05) (Fig. 1). However, at 12 h, the differences on the dry matter, neutral detergent fibre and acid detergent fibre digestibility corn silage in the rumen were not significant (P>0.05) among all the treatments. At 24 h, the neutral detergent fibre and organic matter digestibility of the LB treatment were significantly improved compared with those of the other treatments (P<0.05). Compared with the control condition, all inoculation treatments resulted in improved (P<0.05) dry matter, acid detergent fibre and neutral detergent fibre digestibility; only the organic matter digestibility of the LB treatment (89.62%) was significantly higher than that of the other treatments (P<0.05) at 36 h. The consistency of the differences was poor between 48 and 72 h. At 48 h, the dry matter digestibility of the LB treatment (91.09%) was significantly higher than that of the other treatments (P<0.05). The acid detergent fibre digestibility of the LB and LPLB treatments was significantly higher than that of the other treatments (P<0.05); moreover, the neutral detergent fibre digestibility of the LP and LB treatments was significantly higher than that of the other treatments (P<0.05) and there were no significant differences (P>0.05) on the organic matter digestibility of the experimental conditions. The dry matter digestibility of the LB treatment (91.87%) was significantly improved compared to that of the other treatments (P<0.05), and the acid detergent fibre and neutral detergent fibre digestibility of the LP and LB treatments were significantly improved compared to that of the other treatments (P<0.05) at 72 h.

Table 2: The effect of different treatments on the pH, main microbial composition, CO_2 production and aerobic stability of corn silage after 5 days of open silage jar

Treatments	pH	g/kg	cfu/g wet silage		hour
		CO_2 production	Lactic acid bacteria	Mould	Aerobic stability
Control	3.87 c	5.22 a	6.70	3.08 c	131.71 b
LP	4.01 b	2.43 b	7.48	3.98 b	131.54 b
LB	3.85 c	2.11 b	7.24	3.78 ab	195.58 a
LPLB	4.21 a	1.82 b	7.45	4.26 a	196.21 a
SEM	0.01	0.23	0.16	0.05	35.24
P-value	0.001	0.007	0.345	0.001	0.021

Control = untreated corn silage with no inoculant applied, LP= 1:1: *Lactobacillus plantarum* and *Pediococcus acidilactici* at 1×10^5 cfu/g, LB= *Lactobacillus buchneri* at 1×10^5 cfu/g, LPLB= 1:1:1 *Lactobacillus plantarum, P. acidilactici* and *L. buchneri* at 1×10^5 cfu/g. SEM= standard error of mean. $^{a-c}$ Means within the same columns followed by different letters are significantly different at the 5% level according to the Duncan's multiple range test. n=9

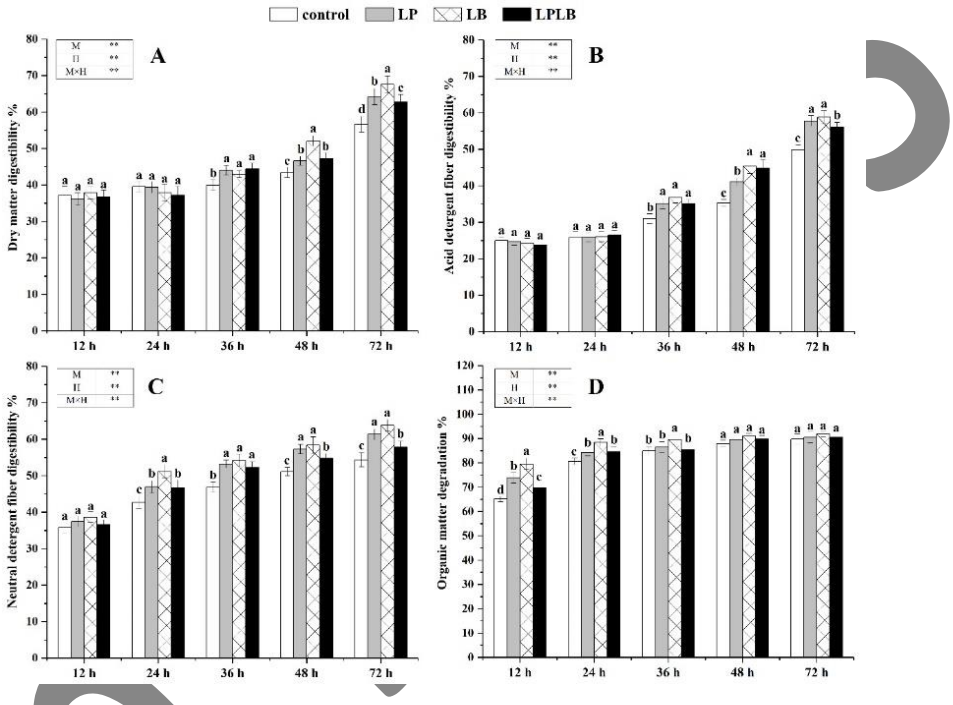

Fig. 1: Effects of different treatments on the dry matter digestibility (A), acid detergent fibre digestibility (B), neutral detergent fibre digestibility (C) and organic matter digestibility (D) of corn silage in rumen. Control = untreated corn silage with no inoculant applied, LP= 1:1 *Lactobacillus plantarum* and *Pediococcus acidilactici* at 1×10^5 cfu/g, LB= *Lactobacillus buchneri* with 1×10^5 cfu/g, LPLB= 1:1:1 *L. plantarum*, *P. acidilactici* and *L. buchneri* at 1×10^5 cfu/g. M= treatment, H= hours, M×H= treatment × hours. Data=mean ± SE. $^{a-c}$ Different letters over the bars at the same time points indicate significant difference at the 5% level according to the Duncan's multiple range test. *= $P<0.05$, **= $P<0.01$. n=9

Two-way analysis of variance showed that treatment (M), time (H) and the interaction of treatment and time (M×H) had significant effects on each index ($P<0.01$) (Fig. 1).

The characteristic parameters of the model of the degradation of dry matter, neutral detergent fibre, acid detergent fibre and organic matter in corn silage in the rumen were analysed. The results showed that the basic content of each nutrient was the same as that at 60 days (Table 1 and 3). The following patterns in the dry matter degradation parameters were observed among the experimental conditions: the *A* of the LB and control conditions was the highest ($P<0.05$); the *B* and *A+B* of all inoculation treatments were lower than that of the control condition ($P<0.05$) and the *C* of the LP treatment was the highest ($P<0.05$). The LP and LB treatments exhibited the most ($P<0.05$) ED of dry matter (ED_{DM}). Regarding the neutral detergent fibre degradation parameters, the *A* of the LB and LP treatments was the highest ($P<0.05$), while the *B* and *A+B* of the LPLB treatment were the highest ($P<0.05$) among the experimental conditions. The ED_{NDF} of the LP and LB treatments was highest of the treatments ($P<0.05$). Regarding the acid detergent fibre degradation parameters, the *A* of the LB and LP treatments was the highest ($P<0.05$), while the other parameters were not significantly different ($P>0.05$) among the treatments. The organic matter degradation parameters had the following results: the *B* of the LP treatment was

Table 3: The effect of different treatments on the digestibility *in rumen* and digestibility coefficients of corn silage

Item	Parameters of digestibility	Treatments				SEM	*P*-value
		Control	LP	LB	LPLB		
Dry matter	Basic content (%)	33.31 c	36.17 b	37.34 a	35.66 b	0.256	<0.001
	A (%)	3.64 a	1.30 b	2.30 a	1.24 b	1.114	0.021
	B (%)	55.93 a	47.66 b	44.25 b	46.43 b	5.659	0.019
	A+B (%)	59.57 a	48.96 b	46.55 b	47.67 b	6.731	0.027
	C (%/h)	0.204 b	0.398 a	0.117 bc	0.095 c	0.108	0.004
	ED_{DM} (%)	3.12 b	4.69 a	3.81 a	2.17 b	1.342	0.007
Neutral detergent fibre	basic content (%)	46.29 a	45.07 b	45.22 b	46.54 a	0.186	0.031
Dry matter	*A* (%)	3.35 b	4.64 a	4.19 a	3.29 b	0.478	0.027
basis	*B* (%)	65.11 d	96.39 c	123.31 b	133.46 a	5.748	0.002
	A+B (%)	68.45 d	101.02 c	127.49 b	136.75 a	5.956	0.006
	C (%/h)	0.019	0.011	0.009	0.010	0.008	0.227
	ED_{NDF} (%)	3.62 b	4.86 a	4.40 a	3.53 b	0.465	0.009
Acid detergent fibre	basic content (%)	26.53 a	25.56 b	24.29 c	25.24 b	0.122	<0.001
Dry matter basis	*A* (%)	0.03 b	0.28 a	0.22 a	0.06 b	0.049	0.006
	B (%)	55.07	57.16	55.58	55.89	0.381	0.224
	A+B (%)	55.10	57.43	55.75	55.95	0.421	0.219
	C (%/h)	0.101	0.104	0.081	0.089	0.019	0.396
	ED_{ADF} (%)	1.24	1.57	1.15	1.14	0.067	0.117
Organic matter	basic content (%)	90.32 c	90.52 b	90.74 a	90.57 ab	0.265	0.009
Dry matter basis	*A* (%)	45.67 b	42.25 c	48.53 a	43.81 bc	0.250	0.024
	B (%)	45.48 a	42.03 b	44.13 a	43.49 ab	0.281	0.031
	A+B (%)	91.15 a	84.28 b	92.65 a	87.29 b	0.525	0.027
	C (%/h)	2.079 b	3.543 ab	4.669 a	2.641 b	1.237	0.035
	ED_{OM} (%)	60.04 b	60.27 b	70.91 a	59.49 b	2.431	0.003

Control = untreated corn silage with no inoculant applied, LP=1:1 *Lactobacillus plantarum* and *Pediococcus acidilactici* at 1×10^5 cfu/g, LB= *Lactobacillus buchneri* at 1×10^5 cfu/g, LPLB= 1:1:1 *Lactobacillus plantarum, P. acidilactici* and *L. buchneri* at 1×10^5 cfu/g. SEM= standard error of mean. [a-c] Means within one row followed by different letters are significantly different at the 5% level according to the Duncan's multiple range test. *A*= Rapid degradation percentage, *B*= Slow degradation percentage, *A+B*=Potential degradation percentage, *C*=degradation rate of *B*, ED= effective degradation. n=9

significantly lower than that of the control and LB treatments ($P<0.05$); the *C* of the LB treatment was significantly higher than that of the control and LPLB treatments ($P<0.05$); and the *A+B* of the LB and control treatments was significantly higher than that of the other treatments ($P<0.05$). Moreover, the *A* of organic matter and ED_{OM} of the LB treatment were significantly higher than those of the other treatments ($P<0.05$).

Discussion

Silage fermentation is a complex and dynamically changing system. According to the results of previous studies, *L. buchneri* alone or in combination with *P. pentosaceus* has a higher pH, greater acetic acid content, lower mould level, and significantly higher lactic acid content than the control treatment when combined treatment of two kinds of LAB (Schmidt and Kung, 2010). Furthermore, the main metabolite of homofermentative LAB in silage fermentation is lactic acid, while the metabolism of heterofermentative LAB not only produces lactic acid but also produces the acetic acid that inhibits mould growth. Lactic acid played an extremely important role in reducing and maintaining low pH in silage (Jungbluth *et al.,* 2016). In this study, the effects of the LPLB treatment on pH, acetic acid and lactic acid content were the same as reported by Schmidt and Kung (2010) and Jungbluth *et al.* (2016). The number of LAB dominates and limits the activity of aerobic microorganisms in the silage fermentation process (Koc *et al*., 2017). This study found that the combined treatment of two kinds of homofermentative LAB (*L. plantarum* and *P. acidilactici*) alone or in combination with heterofermentative LAB (*L. buchneri*) contributed to the increase in the number of LAB after 8 to 35 days of fermentation. The growth of mould was not significantly inhibited as compared to the uninoculated control. Inoculation with *L. buchneri* inhibited the growth of mould to some extent but did not produce significant differences. Therefore, a low dose (1×10^5 cfu/g) of *L. buchneri* may not be sufficient to inhibit the amount of mould during silage fermentation. However, with increasing *L. buchneri* inoculation doses, which can promote the increased production of acetic acid, mould growth is significantly inhibited (Tran *et al.,* 2017). This study analysed aerobic stability 5 days after opening the silage jar and the results indicated that none of the inoculation treatments significantly increased the number of LAB or inhibited the amount of mould; moreover, none of the treatments maintained the acidity of the fermentation system at low pH. However, all inoculation treatments reduced the production of gaseous CO_2, which is mainly produced by aerobic fermentation spoilage microorganisms. At the same time, the aerobic stability time of silage was significantly increased with *L. buchneri* inoculation (LB and LPLB treatments). These results indicated that lactic acid and acetic acid are the main substances that inhibit the growth of fungi. These compounds are not only produced by the three LAB added to corn silage in this experiment; other LAB (*Leuconostoc* spp., *Lactobacillus weiss*, etc.) added to other silage surfaces also

produced lactic acid and acetic acid to inhibit fungal growth (Baek *et al*., 2012). In addition, some LAB strains do not produce high levels of lactic acid but still showed a high rate of fungal inhibition, thus other compounds (cyclic compounds, organic acid, fatty acid and ablastin, etc.) may be produced by LAB metabolism. The production of these compounds was a key factor leading to the phenomena of inhibiting the growth of fungi (Crowley *et al.*, 2013; Gajbhiye and Kapadnis, 2016). *L. buchneri* inhibits the aerobic metamorphism of silage in a manner that mainly depends on acetic acid content (Rabelo *et al*., 2018). However, Akihisa *et al.* (2006) found that adding some of heterofermentative LAB has good aerobic stability with low acetic acid content in *Lolium perenne L.* silage. Therefore, the specific reasons need to be analysed in detail by testing other bacteriostatic substances. Furthermore, in this study, as the fermentation process progressed, the water-soluble carbohydrates and starch gradually decreased. This decrease occurred because the same type of homofermentative LAB consumed carbohydrates to produce the lactic acid that maintained a low pH environment and prevented aerobic spoilage in silage, similar to the results of Wang *et al.* (2017). On the other hand, the pre-fermentation system (0-8 days) improved the activity of enzymes that degrade carbohydrates, thereby promoting the conversion efficiency of carbohydrates to CO_2 (Ávila *et al*., 2008). In addition, heterofermentative LAB dominated late fermentation (15-60 days); these bacteria not only use carbohydrates to produce acetic acid but also use lactic acid to produce acetic acid and a small amount of 1,2-propylene glycol (Tran *et al*., 2017). The role of heterofermentative LAB may be used to reduce carbohydrates to a certain extent. However, the synergistic effect of the homo- and heterofermentative LAB did not slow the loss of carbohydrates.

The ammonium nitrogen content mainly reflected the degree of protein degradation and is an important indicator for evaluating the quality of silage. The amino acids were decomposed into ammonia, hydrothion and amines as a result of the action of undesirable microorganisms when the pH of the fermentation system exceeded 4.2. The degree of degradation was enhanced, and the silage quality deteriorated (Hashemzadeh *et al.*, 2014). In this study, only the LPLB treatment significantly reduced the ammonium nitrogen content on the second day of fermentation; neither the LP nor LB treatment significantly affected the ammonium nitrogen content in the fermentation system. Many previous studies have shown that while the inoculation of the combination homo- and heterofermentative LAB in silage has a certain effect at an inoculation dose greater than 5×10^5 cfu/g, this effect was not significantly different from the uninoculated control (Fanfan *et al.*, 2018). However, protein degradation could be significantly reduced (decreased ammonium nitrogen content) after the inoculation of heterofermentative LAB (4×10^5 cfu/g) (Kleinschmit and Kung, 2006; Reich and Kung, 2010). Therefore, the protein degradation was dependent on the dose of heterofermentative LAB. When inoculated with the combination of homo- and heterofermentative LAB, the ability of heterofermentative LAB to inhibit protein degradation was limited, which may be due to the competition between the different fermentation types of LAB or the low dose of inoculation, making the inhibition mediated by anaerobic *Clostridium* spp. incomplete (Okereke and Montville, 1991). The degree of crude protein content loss was very small in the fermentation process. The crude protein content was the highest with the combined inoculation of *L. plantarum*, *P. acidilactici* and *L. buchneri.* The main reason for this effect was the inoculation of LAB with different types of fermentation; heterofermentative LAB reduced the conversion efficiency of protein to ammonium nitrogen (Weinberg *et al*., 2002; Filya, 2003a, b), while the protein produced by homofermentative LAB increased the crude protein content of the silage system (Rowghani *et al*., 2008; Abdul *et al*., 2017). The dry matter content was particularly important as a basis for the nutrient content of corn silage. In this study, all inoculation treatments improved the dry matter content to only a certain extent; the dry matter content was not dependent on bacteria or on dose, as described by Oliveira *et al.* (2017) and Fanfan *et al*. (2018). The effects of homo- and heterofermentative LAB significantly reduced the neutral detergent fibre and acid detergent fibre contents, respectively. This result may be because the fermentation of different types of LAB produces some feruloyl esterase, which could hydrolyse the ferulic acid ester bond between lignin and hemicellulose, destroying the fibre structure, thereby reducing the fibre content and improving the fibre digestibility of silage (Nsereko *et al*., 2008).

The digestion and utilization of crude fibre by ruminants is mainly carried out by the degradation of microorganisms in the rumen. The apparent digestibility of neutral detergent fibre and acid detergent fibre could be measured to reflect the digestive degradation of rumen microorganisms. In this study, the ED_{DM} and ED_{NDF} of the LP and LB treatments were significantly higher than those of the other treatments (P<0.05). This phenomenon is the same as that reported by Salawu *et al.* (2001) and Aksu and Baytok (2004). The digestibility of dry matter, neutral detergent fibre and acid detergent fibre increased gradually with prolonged time in the rumen (12-72 h), while the digestibility of organic matter increased only at 12-24 h and then remained at approximately 90%. Another study inoculated LAB on *Pisum sativum L.*, *Triticum aestivum L.* and *Oryza sativa L.* silage and obtained the same conclusions as this experiment (Salawu *et al*., 2001; Aksu and Baytok, 2004). In these studies, in each of the different substrate silage substrates, the different fermentation times, LAB types and LAB doses led to changes in the degradation rates of the fermented silage samples in the rumen. However, some studies have shown that the inoculation of *L. plantarum* on the silage of different vegetable residues increased the in vitro digestibility of only some of the vegetable residues (Cao *et al*., 2011), indicating that not all crops or agricultural by-products are suitable for

silage fermentation; controlling the water content and nutrient ratio of the fermented substrate might be the main cause of these different results. In addition, the role of microorganisms, especially LAB, in the fermentation system could not be ignored. *Pedicoccus* sp. played a very important role in the early stage of silage fermentation. After harvesting the silage, the plant was first fermented with *S. faecium* and *Leuconostoc mesenteroides*, followed by more acid-resistant strains such as *L. plantarum*, *L. brevis* and *L. buchneri* (Mcdonald *et al.*, 1991; Yitbarek and Tamir, 2014). In this study, as the rumen retention time of silage corn increased, the digestibility of dry matter, neutral detergent fibre, acid detergent fibre and organic matter increased. Another possible reason was that the addition of each LAB treatment significantly increased the number of LAB in the fermentation system. Therefore, by increasing the number of beneficial LAB in the rumen, the ability to digest nutrients from beneficial bacteria in the rumen was improved. The rumen environment is a relatively cumbersome microbial system in which a variety of microorganisms and factors play an important role. This study did not analyse the rumen microbial diversity and quantity, so further research is needed to better understand the additive effect of LAB on ruminants.

Conclusion

This study found that the inoculation of LAB in corn silage at a low dose has a certain effect on fermentation, aerobic stability, and ruminal digestibility. The combination of homo- and heterofermentative LAB increased the number of LAB in the fermentation process, which was most effective in improving the contents of lactic acid and crude protein. Treatment with *L. buchneri* alone could maintain the fermentation system at a low acidity in the late stage of fermentation (35-60 days). The most marked degradation of neutral detergent fibre and acid detergent fibre was observed when the homo- and heterofermentative LAB were added separately; under the treatments with only homo- or heterofermentative LAB, the digestibility of dry matter and neutral detergent fibre of corn silage in the rumen were also significantly improved. However, compared with the control condition, treatment with only *L. buchneri* significantly increased the digestibility organic matter of corn silage in the rumen and the dry matter content during fermentation. The addition of LAB did not significantly inhibit the growth of mould during the fermentation process and after opening the jar for 5 days, nor did it significantly increase the acetic acid, water-soluble carbohydrates and starch contents during the fermentation process. *L. buchneri* alone or combined with homofermentative LAB improved the aerobic stability time of corn silage.

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