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Original research article

Effects of applying lactic acid bacteria to the fermentation on a mixture of corn steep liquor and air-dried rice straw

Xinxin Li [a], Wenbin Xu [a], Jinshan Yang [a], Hongbo Zhao [b], Chunfang Pan [a], Xue Ding [a], Yonggen Zhang [a, *]

[a] College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, China
[b] Institute of New Rural Development, Northeast Agricultural University, Harbin 150030, China

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ABSTRACT

This study was to determine the fermentation quality of a mixture of corn steep liquor (CSL) (178 g/kg wet basis) and air-dried rice straw (356 g/kg wet basis) after being treated with inoculants of different types of lactic acid bacteria (LAB). The treatments included the addition of no LAB additive (control), which was deionized water; homo-fermentative LAB alone (^{ho}LAB), which was *Lactobacillus plantarum* alone), and a mixture of homo-fermentative and hetero-fermentative LAB ($^{he + ho}$LAB), which were *L. plantarum*, *Lactobacillus casei*, and *Lactobacillus buchneri*. The results showed that the inoculation of the mixture of CSL and air-dried rice straw with $^{he + ho}$LAB significantly increased the concentration of acetic acid and lactic acid compared with the control ($P < 0.05$). The addition of $^{he + ho}$LAB effectively inhibited the growth of yeast in the silage. The concentration of total lactic acid bacteria in the $^{he + ho}$LAB-treated silage was significant higher than those obtained in other groups ($P < 0.05$). The duration of the aerobic stability of the silages increased from 56 h to >372 h. The control group was the first to spoil, whereas the silage treated with $^{he + ho}$LAB remained stable throughout the 372 h period of monitoring. The results demonstrated that the $^{he + ho}$LAB could effectively improve the fermentation quality and aerobic stability of the silage.

1. Introduction

China is an agricultural country with abundant straw resources (Bi et al., 2008). However, only 15% of the rice straw is fed to ruminants; the rest is burnt or buried in the field (Wang et al., 2011). Under natural conditions, fresh rice straws rapidly become air-dried. Thus, it is not a suitable feed for animals because of its low digestibility, poor palatability, high crude fibre, and low protein content. Corn steep liquor (CSL) is a major by-product obtained from the wet-milling industry. It contains a rich complement of organic nitrogen and vitamins, which is capable of replacing yeast extract in a variety of fermentation process (Nascimento et al., 2009). Corn steep liquor has been successfully used in the production of enzymes (Silveira et al., 2001), lactic acid (Agarwal et al., 2006; Liu et al., 2010), and ethanol (Silveira et al., 2001; Saxena and Tanner, 2012). Consequently, air-dried rice straw, as an extensive source of absorbers, can be mixed and ensilaged with CSL effectively. However, the stems of air-dried rice straw have weak natural adhesion to lactic acid bacteria (LAB), thus it is essential to add silage bacterial additives to the mixture of CSL and rice straw to improve the concentration of LAB (Wilkinson, 2005).

Commercial homo-fermentative LAB (^{ho}LAB) inoculants have been developed to ensure rapid and efficient fermentation of water-soluble carbohydrate (WSC) into lactic acid, a rapid decrease in pH, and improved silage conservation with minimal fermentation losses (Huisden et al., 2009; Weinberg et al., 1993). However, such inoculants have also been responsible for decreasing the aerobic stability of silages observed in many studies (Weinberg et al., 1993; Kleinschmit et al., 2005) because antifungal volatile

* Corresponding author.
E-mail address: zhangyonggen@sina.com (Y. Zhang).
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fatty acid (VFA) are lowered and lactic acid is easily oxidized by aerobic microorganisms (MacDonald et al., 1991; Kleinschmit et al., 2005). The aerobic stability of forage with *Lactobacillus buchneri* inoculation can be considerably enhanced by the metabolic activity of converting lactic acid to acetic acid under anaerobic conditions, and the silage could remain cool; thus, it does not spoil as long as 30 d when it is exposed to air (Driehuis et al., 1999; Elferink et al., 2001). Recently, dual-purpose inoculants containing homo-fermentative and hetero-fermentative bacteria have been developed to overcome the limitations of inoculants containing either type of bacteria alone, and the combination of both types of organisms has the potential to improve the speed of fermentation and enhance the aerobic stability (Nishino et al., 2007; Reich and Kung, 2010; Schmidt and Kung Jr, 2010; Conaghan et al., 2010; Kung et al., 2003), but the fermentation of a mixture of CSL and air-dried rice straw with LAB inoculants (*Lactobacillus plantarum*, *L. buchneri* and *Lactobacillus casei*) has not yet been studied.

The present work was to study the effects of ensiling a mixture of CSL and air-dried rice straw with ^{ho}LAB alone or in combination with hetero-fermentative LAB ($^{he\ +\ ho}$LAB) on the fermentation quality and aerobic stability. This experiment was performed by the application of the microorganisms to laboratory silages. The ability to successfully convert a mixture of CSL and air-dried rice straw into a new type of ruminant fermentation feed will promote the improvement in the ecological environment and the cyclic development of the agricultural economy.

2. Materials and methods

2.1. Forage and ensiling

2.1.1. Experimental materials

Rice straws were collected from the Xiangfang Experimental Farm of Northeast Agricultural University (Harbin, China), air-dried to 10% dry matter (DM), and chopped to a theoretical length of 2 to 3 cm. Corn steep liquor obtained from the Cargill Biochemistry Co., Ltd. (Songyuan, China) was used in the study.

2.1.2. Treatments

The CSL (178 g/kg, wet basis) were mixed with 356 g/kg air-dried rice straw (wet basis) thoroughly before the application of the different fermentation inoculants. The mixture was then assigned to one of the following treatments: 1) deionized water, untreated (control); 2) *L. plantarum*, alone (^{ho}LAB); 3) *L. plantarum*, *L. casei*, and *L. buchneri*, a mixture of homo- and hetero-fermentative LAB ($^{he\ +\ ho}$LAB). The application rate of each inoculant to the fresh forage was 1×10^6 cfu/g of fresh matter (FM). The L. *plantarum* strain isolated from a commercial inoculant (Silage. help) was used. The $^{he\ +\ ho}$LAB obtained from Northeast Agricultural University consisted of 2 strains of ^{ho}LAB (*L. plantarum* and *L. casei*) in combination with a ^{he}LAB (*L. buchneri*). In this experiment, the bacterial additive of each group was inoculated in De Man, Rogosa, or Sharpe (MRS) broth for 48 h, and the bacteria were then plated on MRS agar overnight to confirm their viability. Appropriate amounts of the inoculants were then used to achieve the desired application rate. The inoculants were applied at a rate of 50 mL/kg (wet basis) forage with a sprayer. Approximately 416 mL/kg (wet basis) deionized water was sprayed onto the mixture to achieve a final moisture content of 60%. To ensure that the amount of moisture was equal to what was found in the microbial-treated group, the control silage was sprayed with 466 mL/kg (wet basis) deionized water. Approximately 300 g (wet basis) of forage from each treatment were packed into a plastic bag (polyethylene; 400 mm × 500 mm), and all of the bags were sealed with a vacuum sealer and stored indoors for 60 days at ambient temperature (18 ± 2 °C). Duplicate silos for each treatment were opened after 0, 3, 5, 7, 10, 30, 45 and 60 days. The silages were randomly subsampled from several different positions, and then mixed to generate a composite sample for microbiological and chemical analysis. The rest of the bag was subjected to an aerobic stability test after 60 days.

2.2. Chemical and microbial analysis

The silage samples were dried at 65 °C and analysed for DM according to AOAC (1990) procedures. The nitrogen (N) content was measured using the Kjeldahl method (AOAC, 1990). The CP was calculated as N × 6.25. The acid detergent fibre (ADF) and neutral detergent fibre (NDF) values were analysed according to the procedures described by Van Soest et al. (1991) using the Ankom system (Ankom 220 fibre analyser; Ankom) with heat-stable α-amylase. The WSC concentration was measured through the colorimetric method (Dubois et al., 1956). Both fresh and silage juice were extracted by blending 10 g forage (wet basis) in 90 mL of distilled water and storing the mixture for 24 h at 4 °C in a refrigerator (Nishino and Uchida, 1999). The slurry mixture was then filtered through 4 layers of cheesecloth (Xing et al., 2009), and the filtrate was used for pH, ammonia-N, lactic acid and VFA determination. The pH was directly measured using a pH meter (Sartorius Basic pH Meter, Germany). The ammonia-N (NH_3–N) concentration was determined using an ammonia-sensing electrode (Expandable Ion Analyser EA 940, Orion, USA). Samples for VFA analysis were prepared as described by Li and Meng (2006). The concentrations of VFA were analysed using a gas–liquid chromatography (GC, 2010, Tokyo, Japan) equipped with a flame-ionization detector and a free fatty acid phase (FFAP) capillary column (HP-INNOWAX, 30 m × 0.250 mm × 0.25 μm). The lactic acid content was determined using a high-performance liquid chromatography (Waters 600, Tokyo, Japan) following the procedure described by Muck and Dickerson (1987).

Another portion of the fresh silage juices was extracted by blending 10 g forages (wet basis) in 90 mL distilled water for 30 min at ambient temperature, and then filtered through a double layer of cheesecloth. The filtrate was divided into 2 sets of LAB by pour-plating on MRS agar, and the yeasts and moulds were enumerated by pouring the filtrate onto malt extract agar (Oxoid CM0059). The plates were incubated at 37 °C for 48 h, and then numbers of colony-forming units were counted.

All of the chemical analyses were conducted in triplicates, and the results were expressed on a DM basis except that the microbiological data were transformed to log units (% fresh matter), DM content (% fresh matter) and NH_3–N (% total nitrogen).

2.3. Statistical analysis

The data were analysed as a completely randomized design using the ANOVA procedure of SAS 9.2 (SAS Institute, 1999). The results were presented as the mean values and standard error of the means. Differences between treatment means were determined by Duncan's multiple range test method.

3. Results

The chemical compositions of CSL, air-dried rice straw, and their mixture before ensiling are showed in Table 1. The dry matter content of each treatment was adjusted to 40%. The content of CSL (characterized by high protein content) was up to 37% DM, which was favourable for the growth of bacteria. As shown in Table 1, the application of CSL effectively increased the protein content of the dry rice straw to 9.95%. The content of NDF and ADF of dry rice

Table 1
Chemical composition (%) of raw materials before ensiling (DM basis).

Item	Treatment		
	CSL	Rice straw	CSL + rice straw[1]
DM	45.62	89.19	40.00
CP	37.00	3.23	9.95
NDF	0.04	69.09	54.82
ADF	0.06	43.18	34.12
Ash	15.57	14.8	15.41
WSC	6.95	2.9	3.25

CSL = corn steep liquor; NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water-soluble carbohydrate.
[1] The mixture of approximately 416 mL/kg (wet basis) deionized water, 356 g/kg air-dried rice straw (wet basis) and 178 g/kg CSL (wet basis).

straw decreased to 54.82% and 34.12%, respectively. The concentration of WSC in the mixture of CSL and rice straw pre-ensiling was 3.25% DM.

The populations of the LAB and yeast in the mixture of CSL and air-dried rice straw silages after 0-d, 3-d, 5-d, 7-d, 10-d, 30-d, 45-d and 60-d ensiling are showed in Table 2. After 5-d ensiling, the population of LAB in $^{he+ho}$LAB-treated silage increased rapidly from 7.72 $\log_{10}$cfu/g to 9.30 $\log_{10}$cfu/g of FM, which was significantly higher than those of the other two treatments ($P < 0.05$). In contrast, the populations of LAB in control and ^{ho}LAB-treated silage began to decrease after 5 d. After 60-d ensiling, the count of total LAB in $^{he+ho}$LAB-treated silage was significant higher than those in control and ^{ho}LAB-treated silage ($P < 0.05$). All of the 3 treatments showed that the yeast population had a decreasing trend after 5-d ensiling (Table 2). Compared with the control and ^{ho}LAB-treated silage, $^{he+ho}$LAB-treated silage had a significant lower count of yeast existed in during 60-d ensiling ($P < 0.05$). In addition, no mould was found during the 60-d ensiling in all of the samples.

The chemical compositions and fermentation characteristics of a mixture of CSL and air-dried rice straw silages after 60 d of ensiling are showed in Table 3. The silage CP ranged from 10.26% to 10.39%

Table 2
Changes of the population of lactic acid bacteria (LAB) and yeast in corn steep liquor (CSL) and air-dried rice straw silages during 60 days of ensiling ($\log_{10}$cfu/g) [1].

Item	Treatments			SEM	*P*-value
	Control[2]	^{ho}LAB[3]	$^{he+ho}$LAB[4]		
Population of LAB, $\log_{10}$cfu/g					
0 d	6.83^{b}	7.73^{a}	7.72^{a}	0.19	<0.05
3 d	7.45^{c}	7.88^{b}	9.02^{a}	0.30	<0.05
5 d	7.75^{c}	8.00^{b}	9.30^{a}	0.30	<0.05
7 d	7.58^{b}	7.69^{b}	9.06^{a}	0.30	<0.05
10 d	7.28^{b}	7.47^{b}	9.10^{a}	0.37	<0.05
30 d	6.73^{c}	7.04^{b}	8.82^{a}	0.41	<0.05
45 d	6.34^{b}	6.47^{b}	8.98^{a}	0.54	<0.05
60 d	6.04^{c}	6.35^{b}	8.75^{a}	0.54	<0.05
Population of yeast, $\log_{10}$cfu/g					
0 d	6.92	6.94	6.95	0.01	NS
3 d	7.85^{b}	7.97^{a}	7.49^{c}	0.09	<0.05
5 d	7.76^{a}	7.93^{a}	7.46^{c}	0.09	<0.05
7 d	7.45^{a}	7.30^{b}	6.92^{c}	0.10	<0.05
10 d	7.32^{a}	7.03^{b}	5.95^{c}	0.26	<0.05
30 d	6.69^{a}	5.95^{b}	2.15^{c}	0.89	<0.05
45 d	6.15^{a}	5.14^{b}	0.00^{c}	1.21	<0.05
60 d	6.02^{a}	5.02^{b}	0.00^{c}	1.18	<0.05

SEM = standard error of the mean; NS = not significant.
[a, b, c] Within a same row, means with different letters differ ($P < 0.05$).
[1] Values are presented as the mean values ($n = 3$).
[2] Control means no additive applied.
[3] ^{ho}LAB means homo-fermentative LAB alone (*L. plantarum*).
[4] $^{he+ho}$LAB means a mixture of ^{ho}LAB and hetero-fermentative LAB (*L. plantarum*, *L. casei*, *L. buchneri*).

Table 3
Chemical compositions and fermentation characteristics of corn steep liquor (CSL) and air-dried rice straw silages after 60 days of ensiling [1].

Item	Treatments			SEM	*P*-value
	Control[2]	^{ho}LAB[3]	$^{he+ho}$LAB[4]		
Chemical compositions					
DM, %	39.28^{c}	39.71^{b}	39.98^{a}	0.13	<0.05
CP, % DM	10.26	10.32	10.39	0.04	NS
NDF, % DM	59.38^{b}	60.62^{a}	61.24^{a}	0.31	<0.05
ADF, % DM	37.7	37.82	37.09	0.24	NS
WSC, % DM	0.63^{a}	0.57^{a}	0.46^{b}	0.03	<0.05
WSCL, %	80.74^{b}	82.39^{b}	85.82^{a}	0.78	<0.05
Fermentation characteristics					
pH	4.45^{b}	4.43^{b}	4.52^{a}	0.02	<0.05
NH3–N	0.07^{c}	0.13^{b}	0.16^{a}	0.02	<0.05
NH3–N, % of total N	4.40^{c}	7.83^{b}	9.81^{a}	1.01	<0.05
AA, % DM	1.13^{c}	1.42^{b}	2.31^{a}	0.23	<0.05
LA, % DM	4.02^{b}	5.11^{a}	5.62^{a}	0.31	<0.05
LA:AA, % DM	3.56^{a}	3.60^{a}	2.43^{b}	0.25	<0.05

SEM = standard error of the mean; NS = not significant; NDF = neutral detergent fibre; ADF = acid detergent fibre; WSC = water-soluble carbohydrate; WSCL = WSC loss; LA = Lactic acid; AA = acetic acid.
[a, b, c] Within a same row, means with different letters differ ($P < 0.05$).
[1] Values are presented as mean values ($n = 3$).
[2] Control means no additive applied.
[3] ^{ho}LAB means homo-fermentative LAB alone (*L. plantarum*).
[4] $^{he+ho}$LAB means a mixture of homo-fermentative LAB and hetero-fermentative LAB (*L. plantarum*, *L. casei*, *L. buchneri*).

and there was no significant difference between groups. Inoculation with $^{he+ho}$LAB had no effects on the ADF content of silages compared with inoculation with ^{ho}LAB or control silages; however, it had a higher concentration of NDF than the other 2 groups ($P < 0.05$). The residual WSC was significantly lower ($P < 0.05$) in the $^{he+ho}$LAB-treated silage compared with the amount in the ^{ho}LAB-inoculated or control silages. In addition, $^{he+ho}$LAB had a higher concentration of WSCL than the other two groups ($P < 0.05$). Moderately higher pH value of the $^{he+ho}$LAB-treated silage was detected compared with the pH values of the control and ^{ho}LAB-treated silage ($P < 0.05$). After 60 days of ensiling, the silage inoculated with $^{he+ho}$LAB had a higher content of NH_3–N (fresh matter) compared with the ^{ho}LAB-treated and control silages ($P < 0.05$). As shown in Table 3, the silage inoculated with $^{he+ho}$LAB resulted in a higher ratio of NH_3–N to total nitrogen compared with the control and ^{ho}LAB-treated silage ($P < 0.05$). The highest level of acetic acid was observed in the $^{he+ho}$LAB-treated silage (2.31%), and this level was significantly higher in this treatment than in ^{ho}LAB-treated or control ($P < 0.05$). The control group had the lowest ($P < 0.05$) concentration of lactic acid (4.02% DM) compared with the ^{ho}LAB (5.11% DM) and $^{he+ho}$LAB-treated silages (5.62% DM). As shown in Table 3, the ratios of lactic acid to acetic acid for the control and ^{ho}LAB-treated silages were 3.56 and 3.60, respectively. In contrast, the ratio for $^{he+ho}$LAB-treated silage was 2.43. No butyric acid was detected in this experiment.

The aerobic stability of the silage is presented in Fig. 1. The aerobic stability of the silages ranged from 56 h to >372 h. The control group was the first to spoil, whereas the silage treated with $^{he+ho}$LAB remained stable throughout the 372-h period of monitoring.

4. Discussion

The growth of LAB via fermentation requires adequate WSC supplement (MacDonald et al., 1991), and low levels may restrict fermentation process of it. The initial concentration of WSC is sufficient to ensure adequate ensiling (Haigh and Parker, 1985). Hetero-fermentative bacteria *L. buchneri* could generate energy by

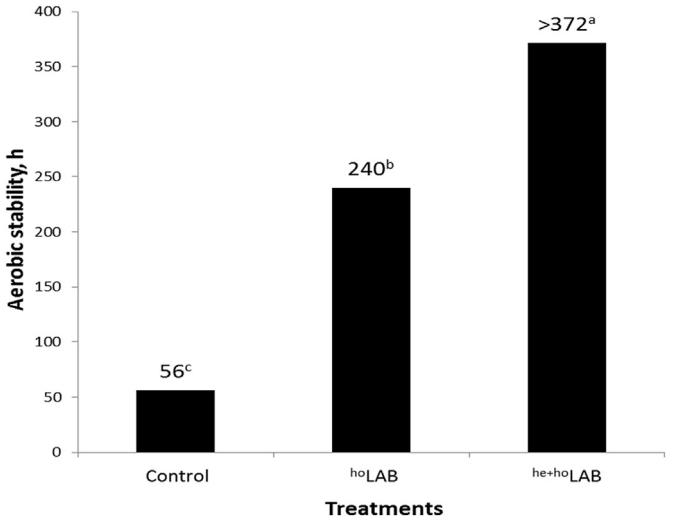

Fig. 1. Effects of lactic acid bacteria (LAB) inoculants on the aerobic stability (hours for the feed temperature to increase 2 °C above ambient temperature) of silages, a mixture of corn steep liquor (CSL) and air-dried rice straw silages, after 60 days of ensiling. Treatments: Control means no LAB additive; ^{ho}LAB means homo-fermentative lactic acid bacteria alone (*Lactobacillus plantarum*); $^{he + ho}$LAB means a mixture of ^{ho}LAB and hetero-fermentative LAB (*Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus buchneri*). $^{a, b, c}$ Above a bar, means with different superscripts differ ($P < 0.05$).

fermenting sugars, which results in the increase of their numbers during the initial phase of fermentation (Schmidt et al., 2009). The addition of CSL for the air-dried rice straw silage increases the available N source for LAB growth; however, high viscosity and some inhibitory component in CSL could also inhibit the growth of it (Agarwal et al., 2006). The addition of ^{ho}LAB in silages did not effectively promote the fermentation process. The decrease in yeast over time was partly due to the growth of LAB, especially, *L. buchneri* in $^{he + ho}$LAB-treated silages. Driehuis et al. (1999) reported that *L. buchneri* could inhibit the growth of yeast by the production of acetic acid both during ensiling and after exposure to air.

The content of CP was unaffected by treatments with different fermentation types of microbial inoculants. This finding was in agreement with the addition of *L. buchneri* to barley silage (Kung and Ranjit, 2001). Hetero-fermentative bacteria of *L. buchneri* in $^{he + ho}$LAB consumes more nutrients during fermentation than homo-fermentative bacteria (Guo et al., 2013), which results in greater NDF concentration on a DM basis in the $^{he + ho}$LAB-treated silage. Moreover, the inoculation of the silages with *L. buchneri* could result in a lower concentration of WSC in the silage (Kung and Ranjit, 2001). The significantly low residual WSC in the $^{he + ho}$LAB-treated silage indicates that WSC is better utilized by the fermentation bacteria in $^{he + ho}$LAB.

The higher pH value of $^{he + ho}$LAB-treated silage was partly due to the production of acetic acid from lactic acid by *L. buchneri* (Elferink et al., 2001). Kleinschmit and Kung Jr (2006) reported that the addition of *L. buchneri* to silages always resulted in a higher pH value. It was proposed that the higher concentration of NH_3–N, as a result of inoculation with *L. buchneri*, may contribute to the increase of pH as a result of $^{he + ho}$LAB addition (Driehuis et al., 2001). Although the addition of classical homo-fermentative acid bacteria to silage often resulted in a decrease in NH_3–N (Muck and Kung Jr, 1997; Conaghan et al., 2010), the addition of the homo-fermentative bacteria *L. plantarum* in $^{he + ho}$LAB could not prevent the increase induced by hetero-fermentative bacteria *L. buchneri*. Some other studies also reported that addition of *L. buchneri* increased the NH_3–N content in alfalfa (Kung et al., 2003; Schmidt et al., 2009) and grass silage (Driehuis et al., 2001). The NH_3–N concentrations of total nitrogen in all of the experimental groups were less than 10%, which demonstrated that the N in silages were well-preserved (McDonald et al., 2002). Acetic acid has been reported as a potent inhibitor of fungi, and plays an active role in aerobic deterioration (MacDonald et al., 1991). Driehuis et al. (1999) and Elferink et al. (2001) reported that *L. buchneri* can produce acetic acid from a novel fermentation of lactic acid under anaerobic conditions. The lactic acid contents of all groups were higher than that (2%) estimated for good-quality silages (Kılıc, 1986). This higher concentration of lactic acid in ^{ho}LAB and $^{he + ho}$LAB can be explained by the addition of the ^{ho}LAB, which could ensure rapid and vigorous fermentation by promoting the production of lactic acid (Muck and Kung Jr, 1997). Kung and Stokes (2001) reported that the ratio of a lactic acid to acetic acid, that was more than 3:1, was an indication of a homolactic dominant fermentation. As shown in Table 2, both ratios of the control and ^{ho}LAB-treated silage were more than 3:1. This result suggests that the fermentation with $^{he + ho}$LAB is dominated by hetero-fermentative bacteria *L. buchneri*, which can also well explain the high NH_3–N, pH and acetic acid in the $^{he + ho}$LAB-treated silage.

It has been reported that residual WSC content, the concentration of lactic acid and antifungal VFA in silages are associated with aerobic spoilage (Weinberg et al., 1993). The residual WSC in the control was higher than those in other groups, and the concentration of acetic acid in the control was the lowest. Thus, the duration of its aerobic stability was short. Silage treated with ^{ho}LAB showed lower stability, and heating appeared earlier in this group than in the $^{he + ho}$LAB-treated silage. This production of acetic acid by *L. buchneri* considerably enhanced the aerobic stability of $^{he + ho}$LAB, and the silage could remain unheated for over 372 h. In some studies, corn silage treated with *L. buchneri* did not spoil throughout 792-h (Driehuis et al., 1999) and 900-h (Ranjit and Kung Jr, 2000) aerobic exposure.

In conclusion, the present study demonstrated that the treatment of a mixture of CSL and air-dried rice straw with an inoculant containing a blend of ^{ho}LAB and ^{he}LAB may be a promising means of improving the fermentation quality and aerobic stability of silages.

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