Chemical composition, fermentative losses, and microbial counts of total mixed ration silages inoculated with different *Lactobacillus* species

Rasiel Restelatto,* Charles O. Novinski,* Lucelia M. Pereira,* Eduardo P. A. Silva,* Denise Volpi,*
Maity Zopollatto,* Patrick Schmidt,* and Antonio P. Faciola^{†1,✉}

*Department of Animal Science, Federal University of Paraná, Curitiba 80035050, Brazil; and [†]Department of Animal Sciences, University of Florida, Gainesville 32611, FL

ABSTRACT: The objective of this study was to evaluate the effects of *Lactobacillus* inoculants on fermentation, losses, and aerobic stability of a total mixed ration (TMR) silage. A TMR, formulated to meet the requirements of dairy cows producing 25 kg of milk/d, was applied with the following treatments prior to ensiling: 1) Control (CON), 2) *Lactobacillus buchneri* (10^5 cfu/g of fresh forage; LB), and 3) *Lactobacillus plantarum* (10^5 cfu/g of fresh forage; LP). TMR silages were ensiled for 15 and 60 d in silos equipped with an apparatus for determination of gravimetric DM, gas, and effluent losses. The experiment was performed in a complete randomized design with a 3×2 factorial arrangement of the treatments, with 5 replicates per treatment. Chemical changes, microbial counts, fermentation profile, and aerobic stability were measured after opening the silos. Data were submitted to ANOVA, and means were compared by Tukey and *T*-test and statistical

significance was declared at $P \leq 0.05$. After 15 d of ensiling, the inclusion of inoculant decreased NDF ($P < 0.05$) and butyric acid concentrations ($P < 0.05$) in TMR. LP had the lowest aerobic stability ($P < 0.05$) and the greatest loss of DM ($P < 0.03$). Ensiling for 60 d increased ammonia nitrogen ($\text{NH}_3\text{-N}$), lactic acid bacteria (LAB), aerobic stability, and concentrations of lactic and acetic acid ($P < 0.01$) and lowered ($P < 0.02$) total fermentation losses compared to 15 d across all treatments. After 60 d of ensiling, LP lowered pH to the greatest extent. Treatment had no effect on concentrations of DM, CP, ADF, ash, and EE, as well as in vitro DM digestibility. In conclusion, inoculants containing LP or LB did not improve fermentation profile, did not prolong the aerobic stability, nor reduced losses. Furthermore, the 15-d ensiling period was insufficient for adequate bacterial activity.

Key words: heterofermentative additive, lactic acid bacteria, *Lactobacillus buchneri*, *Lactobacillus plantarum*, volatile compound, yeast

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INTRODUCTION

The use of microbial inoculants in forage silages (corn, sorghum, grasses, etc.) aims to improve fermentation and aerobic stability (Windle and Kung, 2016). According to Muck (2013), lactic acid bacteria (LAB) are the main

group of microorganisms that act in the forage fermentation process and have the ability to produce lactic acid as their main fermentation product.

From the 6 LAB genera typically involved in the forage fermentation process (*Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Lactococcus*, and *Leuconostoc*), *Lactobacillus* has been shown to be efficient in dominating the epiphytic population in the silage material, producing lactic acid to rapidly decrease the pH of the

¹Corresponding author: afaciola@ufl.edu

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material, which limits *Clostridium* activity that is responsible for butyric fermentation (Muck, 2010, Dunière et al., 2013, Liu et al., 2016). *Lactobacillus plantarum* is used as a microbial additive in traditional silages because it has vigorous growth, causing a rapid pH reduction and grows well on low moisture substrates (Muck, 2010). It is classified as facultative heterofermentative bacteria (Kung, 2009, Muck et al., 2018). *Lactobacillus buchneri* is an obligatory heterofermentative microbial additive widely used in silages because it has the ability to convert lactic acid to acetic acid and 1,2-propanediol in the absence of O₂, which may inhibit fungi and yeasts growth (Oude Elferink et al., 2001).

Lactic acid bacteria are widely used in forage silages (Zopollatto et al., 2009, Oliveira et al., 2017); however, their use in TMR silages in which forages, cereals, minerals, vitamins, and additives are mixed together is still scarce. Liu et al. (2016) evaluated the effects of LP and fibrolytic enzymes on fermentation quality in TMR silages ensiled for 60 d and concluded that the combination of these inoculants yielded the highest lactic acid concentration, thus resulting in the lowest pH and improved fermentation quality of TMR silages. Nishino et al. (2004) found that inoculation of LB in TMR silage can inhibit aerobic spoilage and decrease yeast populations when stored for 60 d but not 10 d. Despite its outstanding effect on aerobic stability, the anaerobic conversion of lactic acid to acetic acid by LB does not occur immediately and needs about 30 to 60 d to become apparent (Muck et al., 2018).

We hypothesized that the use of the additive LP would improve the fermentation profile of TMR ensiled for 15 d because of the rapid pH reduction and that LB would increase the aerobic stability of TMR ensiled for 60 d because of an increase in acetic acid concentration. Our objectives were to evaluate the effects of these inoculants on fermentation traits, storage losses, microbial counts, as well as the aerobic stability of TMR silages, ensiled for 15 or 60 d.

MATERIALS AND METHODS

Experimental Design and Treatments

Ingredients used to formulate the TMR are presented in Table 1. The TMR was formulated to meet the nutritional requirements of a lactating dairy cow producing 25 kg of milk/d NRC (2001). The corn silage and ryegrass haylage used in the TMR did not contain any inoculants or additives. Chemical composition and microbial counts of TMR before

Table 1. Ingredient composition in total mixed ration (TMR) before ensiling

Ingredients	TMR (% DM basis)
Corn silage	44.9
Ryegrass haylage	19.5
Ground corn	13.6
Soybean meal (46%)	9.34
Corn gluten meal ¹	9.27
Sodium bicarbonate	1.03
Mineral mix ²	1.03
Sodium chloride	0.51
Urea	0.41
Calcitic limestone	0.41

¹Crude protein (24.4% DM), crude fiber (9.4% DM), starch (8.9% DM), ash (7% DM).

²Sulfur (0.7% DM), sodium (0.04% DM), chlorine (0.35% DM), iron (144 ppm), manganese (23 ppm), zinc (85 ppm), copper (7.5 ppm), molybdenum (1.7 ppm), cationic-anionic difference in diet (5.8 meq/100 gdm),

ensiling are presented in Table 2. Dietary ingredients were mixed in a vertical stationary mixer (Trioliet model 16000L Oldenzaal, Netherlands) for 7 min and were subsequently distributed in 100 kg batches over plastic sheeting for inoculant application and then ensiled. Treatments were: Control (without inoculant; CON), *Lactobacillus buchneri* CNCM I-4323 (10⁵ cfu/g; Lallemend Animal Nutrition; LB), and *Lactobacillus plantarum* N2072 (10⁵ cfu/g; Lallemend Animal Nutrition; LP). All treatments were stored for 15 and 60 d.

Each inoculant was diluted in 500 mL deionized water in a plastic bottle and sprayed manually over 100 kg batches of the TMR placed on plastic sheeting. In the control treatment, 500 mL of deionized water with no inoculant was applied. The dose of the inoculants LB and LP was determined according to Driehuis et al. (1999) and Weinberg and Muck (1996), respectively. Just before ensiling, a subsample of 0.3 kg was collected for the analysis of pH, DM, chemical composition, and microbial counts.

Ensiling Process and Sample Collections

The TMR was compacted in 8.8-L experimental PVC silos with a packing density of 350 kg DM/m³, with 5 replicates per treatment. The silos were equipped with an apparatus for the gravimetric determination of total DM losses (TDML), gas losses (GLoss), and effluent losses (ELoss) (Jobim et al., 2007). Additionally, the volume of gas produced during fermentation was measured using a system composed of a 1-L graduated low-density polyethylene beaker (Ø—6.5 cm; 43 cm long)

Table 2. Chemical composition, pH, and microbial counts of total mixed ration (TMR) before ensiling

Items	CON $\pm$ SD ⁴	LB $\pm$ SD ⁴	LP $\pm$ SD ⁴
pH	4.76 $\pm$ 0.03	4.82 $\pm$ 0.08	4.87 $\pm$ 0.08
<i>Chemical composition (%)</i>			
DM	41.9 $\pm$ 0.02	41.8 $\pm$ 0.05	41.7 $\pm$ 0.17
CP	15.5 $\pm$ 0.15	15.6 $\pm$ 0.17	16.0 $\pm$ 0.26
NDF	38.1 $\pm$ 0.46	37.6 $\pm$ 0.22	37.9 $\pm$ 0.27
ADF	19.1 $\pm$ 0.56	19.0 $\pm$ 0.64	19.1 $\pm$ 0.27
IVDMD ¹	82.6 $\pm$ 0.85	84.0 $\pm$ 0.36	83.9 $\pm$ 1.01
Starch	31.5 $\pm$ 0.19	31.0 $\pm$ 0.24	30.7 $\pm$ 0.29
EE	2.9 $\pm$ 0.03	2.9 $\pm$ 0.08	2.9 $\pm$ 0.07
Ash	7.5 $\pm$ 0.03	7.7 $\pm$ 0.23	7.5 $\pm$ 0.18
NH ₃ -N (%TN) ²	15.7 $\pm$ 0.06	15.6 $\pm$ 0.31	15.4 $\pm$ 0.40
<i>Microbial counts (log cfu/g)</i>			
LAB ³	5.3 $\pm$ 0.08	5.5 $\pm$ 0.22	5.6 $\pm$ 0.10
Yeasts	4.9 $\pm$ 0.17	5.0 $\pm$ 0.03	5.2 $\pm$ 0.04
Molds	4.0 $\pm$ 0.10	3.8 $\pm$ 0.35	3.9 $\pm$ 0.12

CON = Control (without inoculant); LB = *Lactobacillus buchneri* CNCM I-4323 (10^5 cfu/g of fresh forage); LP = *Lactobacillus plantarum* N2072 (10^5 cfu/g of fresh forage).

¹In vitro dry matter digestibility.

²Ammonia nitrogen as percent of total nitrogen.

³Lactic acid bacteria.

⁴Standard deviation.

connected to each silo by a silicone hose passing through a 3-way stop tap. In the bottom of the beaker, we made a 2-mm hole and a hollow metal pin was inserted to connect the hose. This beaker was maintained with the opening face down, immersed in water, to avoid any gas loss (Souza, 2015). The direct measurement of gas volume (GV) was taken several times per day, and the gas was released after recording the volume. Total GV was the sum of all measurements of each silo during the fermentation period.

After 15 or 60 d of ensiling, the silos were weighed, opened, and the TMR silage removed, homogenized, and sampled. Two subsamples from each silo were collected for the determination of DM content (method number 934.01; AOAC, 1990) and pH as previously mentioned.

Another 2 subsamples of each silo were used to evaluate aerobic stability (Kung and Ranjit, 2001). One sample (1 kg) was kept in a 4-L bucket for measuring daily pH for nine d with a digital pH meter (PG 1400, Gehaka - Brazil) according to Kung et al. (2000). The other sample (3 kg) was kept in a 20-L bucket and its temperature was measured every 30 min during nine d via dataloggers (USB-EL1 - Lascar Inc. Savannah, GA) inserted in the center of the mass. Samples were weighed at the end of the trial to estimate the total DM loss during aerobic exposure (TDMLas). The breakdown of aerobic stability (AS) was established when the temperature of the material raised 2 °C above room temperature (25 ± 1 °C).

Chemical and Microbial Analyses

The samples collected before and after the ensiling periods were dried in an oven with forced ventilation at 55 °C for 72 h then individually ground through a 1 mm screen in a Willey mill (Model #2, Arthur H. Thomas Co., Philadelphia, PA), and CP was determined by the DUMAS method (FP-528, Leco, combustion N analyzer, Leco Instruments Inc., St. Joseph, MI), according to Wiles et al. (1998), ash (method number 924.05; AOAC, 2012), EE (method number 920.39; AOAC, 2012), NDF, and sequential analysis of ADF, treated with thermostable alpha-amylase and sodium sulphite according to Mertens (2002), modified for the Ankom A²⁰⁰ “Fiber Analyzer” (Ankom Technology, Macedon, NY).

The in vitro dry matter digestibility (IVDMD) was performed according to Tilley and Terry (1963) and adapted to ANKOM (Holden 1999). The ash correction was obtained after 4 h in the furnace at 600 °C. The starch was analyzed following the enzymatic procedure (Demiate et al., 2001).

The NH₃-N was determined by calorimetry methodology as described by Chaney and Marbach (1962) adapted for silage samples. Microbial count was conducted in an aqueous extract as described by Kung et al. (1984). Briefly, the aqueous extract used for plating was prepared in sterile plastic bags containing 25 g of fresh sample and 225 mL of 25% Ringer solution. The material was homogenized

for 4 min in a Stomacher shaker (Marconi-MA 440/CF, São Paulo, Brazil) and filtered with 4 layers of cheesecloth. The aqueous extract was submitted to serial dilutions (10 times) in 25% Ringer solution and plated in 3M Petrifilm. The count of lactic acid bacteria was performed in Petrifilm AC, after incubation at 32 °C for 48 h in anaerobic jars. The counts of yeast and moulds were performed in Petrifilm YM after incubation at 25 °C for 72 and 120 h, respectively.

The concentrations of ethanol, acetic acid, propionic acid, and butyric acid in TMR silages were determined by gas phase chromatography according to Erwin et al. (1961), and lactic acid was quantified using the colorimetric method according to Pryce (1969).

Statistical Analysis

The experiment was performed in a complete randomized design with a 3×2 factorial arrangement of treatments. The experimental factors were inoculants (TMR silage with or without inoculants) and storage periods (15 or 60 d). There were 5 replicates per treatment, totaling 30 experimental units. Data distribution and the homogeneity were evaluated, and log transformation was performed when data were not normally distributed (lactic acid bacteria, yeasts, and molds).

Analysis of variance was used for residual analysis using GLM procedure of SAS (Statistical Analysis System, version 9.2, SAS Inst., Inc., Cary, NC (SAS, 2001)). The statistical model used for the analysis was the following:

$$Y_{ij} = \mu + T_i + P_j + TP_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the response, μ is the overall mean; T_i is the effect of treatment, P_j is the effect of the ensiling periods, TP_{ij} is the interaction between treatment and ensiling periods, and ε_{ij} is the error. Tukey and T -test were used to compare treatment means, and significance declared at $P \leq 0.05$. The effects of *Lactobacillus* inoculants on temperature and pH of TMR silage exposed to air after opening were evaluated by polynomial regression analysis, using REG procedure of SAS (Statistical Analysis System, version 9.2, SAS Inst., Inc. [SAS, 2001]).

RESULTS

Dry matter, CP, ADF, IVDMD, ash, EE, and starch content of TMR silages were not affected by treatment, the ensiling periods, or their interaction (Table 3). However, NDF had a significant

interaction ($P < 0.01$) between treatment and ensiling periods (Treatment $\times$ Period), in which CON ensiled for 15 d had the greatest NDF values. $\text{NH}_3\text{-N}$ of TMR silage was lower for TMR ensiling for 15 d compared with 60 d ($P < 0.01$) (Table 3).

For the 15 d of ensiling period, the inoculants did not reduce TMR pH, however, when the TMR were ensiled for 60 d, there was an interaction (Treatment $\times$ Period) ($P < 0.01$), and the LP had the lowest pH value (4.29) when compared with others treatment (4.51 and 4.39 for CON and LB, respectively) (Table 4). Concentration of lactic acid, acetic acid, and ethanol were affected by ensiling periods ($P < 0.01$) and the 15 d opening period had the lowest values of lactic acid (2.5%), acetic acid (2.4%), and ethanol (0.4% DM) when compared to 60 d of ensiling period (3.7, 2.7, and 0.6% for lactic acid, acetic acid, and ethanol, respectively). The concentrations of propionic acid were not affected by treatments, ensiling periods, or their interactions. For butyric acid concentrations, there was an interaction ($P < 0.01$) of treatment and ensiling periods and the control treatment with 15 d had the highest concentration (0.03%) regarding *Lactobacillus* treatment (0.02%) or 60-d stored treatments (0.02%).

There was an ensiling period effect for GV, TDML, GLoss, and ELoss ($P < 0.01$). TMR silage that was opened at 15 d had greater TDML and GLoss but lower GV and ELoss compared with 60 d ensiling period (Table 5). No treatment effect was observed on losses; however, a trend ($P = 0.07$) was detected for greater effluent in inoculated TMR silages (1.1 kg/t) compared to the control (0.8 kg/t).

Lactic acid bacteria (LAB) and yeast counts were affected by ensiling periods ($P < 0.01$), and the 15 d ensiling period had the lowest LAB and the greatest yeast count. The molds on the TMR silage were not influenced by inoculants, ensiling periods and their interaction (Table 6).

There was an interaction of treatment and ensiling periods on the AS and TDMLs. *Lactobacillus plantarum* had the shortest time ($P < 0.01$) to break AS (50 h) and the greatest TDMLs ($P < 0.03$) (23%) when TMR was ensiled for 15 d, when compared to LB (67.6 h and 18%) and to CON (64.4 h and 18%). However, when the TMR were ensiled for 60 d the AS and TDMLs were similar among treatments, and improved when compared to TMR ensiled for 15 d. For TMR silage with 15 d ensiling period (Fig. 1A), there was an interaction of treatment and ensiling periods on temperature ($P < 0.01$). *Lactobacillus plantarum* was the first to spoil (50 h) compared to the CON (64 h) and LB

Table 3. Effects of *Lactobacillus* inoculants on chemical composition of total mixed ration (TMR) silages

Items	Ensiling period, days	CON	LB	LP	SEM ³	P-value ⁴		
						Treatment	Period	Treatment × Period
DM, %	15	41.4	41.5	41.5	0.05	0.57	0.07	0.82
	60	41.6	41.6	41.5	0.04			
CP, %	15	15.5	15.7	15.5	0.11	0.93	0.12	0.42
	60	15.8	15.6	15.8	0.07			
NDF, %	15	38.3 ^{Aa}	35.2 ^{Ab}	35.4 ^{Ab}	0.39	<0.01	0.08	<0.01
	60	36.3 ^{Ba}	35.7 ^{Aa}	35.7 ^{Aa}	0.17			
ADF, %	15	18.9	18.5	18.4	0.09	0.13	0.13	0.36
	60	18.5	18.3	18.4	0.11			
IVDMD, % ¹	15	84.4	83.9	83.3	0.20	0.16	0.84	0.41
	60	84.0	83.9	83.8	0.22			
ASH, %	15	7.4	7.5	7.5	0.03	0.72	0.47	0.29
	60	7.4	7.5	7.4	0.03			
EE, %	15	2.9	2.9	2.9	0.02	0.88	0.40	0.44
	60	2.9	2.9	2.9	0.03			
NH ₃ -N, %TN ²	15	16.0	15.9	16.0	0.10	0.85	<0.01	0.72
	60	16.6	16.5	16.3	0.15			
Starch, %	15	30.6	31.0	30.3	0.18	0.26	0.79	0.25
	60	30.1	30.8	30.8	0.18			

CON = Control (without inoculant); LB = *Lactobacillus buchneri* CNCM I-4323 (10⁵ cfu/g of fresh forage); LP = *Lactobacillus plantarum* N2072 (10⁵ cfu/g of fresh forage).

¹In vitro DM digestibility.

²Ammonia nitrogen as percent of total nitrogen.

³Standard error of mean; capital letter (A; B) are the period comparison within the treatment (column); for the period comparison the *T*-test was used; lowercase letters (a; b) are the comparison of the treatments within the period in line; the Tukey test was used for treatment comparisons.

⁴*P*-value was considered significant when ≤ 0.05; treatment = inoculants, period = ensiling period, and treatment × period = interactions between treatment and period.

Table 4. Effects of *Lactobacillus* inoculants on pH and fermentation products of total mixed ration (TMR) silages

Volatile compounds, %	Ensiling period, days	CON	LB	LP	SEM ¹	P-value ²		
						Treatment	Period	Treatment × Period
pH	15	4.70 ^{Aa}	4.68 ^{Aa}	4.70 ^{Aa}	0.01	< 0.01	<0.01	<0.01
	60	4.51 ^{Ba}	4.39 ^{Bb}	4.29 ^{Bc}	0.02			
Lactic acid	15	2.5	2.4	2.6	0.06	0.24	<0.01	0.94
	60	3.8	3.6	3.8	0.10			
Acetic acid	15	2.4	2.5	2.3	0.06	0.12	<0.01	0.96
	60	2.7	2.8	2.6	0.05			
Propionic acid	15	0.3	0.3	0.3	0.01	0.23	0.19	0.50
	60	0.3	0.3	0.3	0.01			
Butyric acid	15	0.03 ^{Aa}	0.02 ^{Ab}	0.02 ^{Ab}	0.01	<0.01	<0.01	<0.01
	60	0.02 ^{Ba}	0.02 ^{Aa}	0.02 ^{Aa}	0.01			
Ethanol	15	0.4	0.4	0.4	0.01	0.44	<0.01	0.82
	60	0.6	0.5	0.6	0.02			

CON = Control (without inoculant); LB = *Lactobacillus buchneri* CNCM I-4323 (10⁵ cfu/g of fresh forage); LP = *Lactobacillus plantarum* N2072 (10⁵ cfu/g of fresh forage).

¹Standard error of mean; capital letter (A; B) are the period comparison within the treatment (column); for the period comparison the *T*-test was used. Lowercase letters (a; b) are the comparison of the treatments within the period in line; the Tukey test was used for treatment comparisons.

²*P*-value was considered significant when ≤ 0.05; treatment = inoculants, period = ensiling period, and treatment × period = interactions between treatment and period.

(68 h); however, for the TMR silage ensiled for 60 d (Fig. 1B), no heating was detected during 216 h of air exposure, regardless of inoculants used. The

pH pattern of TMR silages exposed to air for 216 h (Fig. 1C and D) were similar to the temperature pattern for treatment and ensiling period.

Table 5. Effects of *Lactobacillus* inoculants on gas volume (GV) produced and fermentation losses of total mixed ration (TMR) silages

Items ¹	Ensiling period, days	CON	LB	LP	SEM ²	P-value ³		
						Treatment	Period	Treatment × Period
GV, L/t of DM	15	385	341	353	8.57	0.43	<0.01	0.19
	60	1265	1294	1372	29.2			
TDML, %	15	1.4	1.0	0.9	0.10	0.89	<0.02	0.54
	60	0.6	0.5	0.8	0.12			
GLoss, %	15	1.2	0.9	0.7	0.14	0.28	<0.01	0.12
	60	0.5	0.5	0.6	0.12			
ELoss, kg/t of FF	15	0.8	0.8	0.9	0.05	0.07	<0.03	0.23
	60	0.9	1.4	1.2	0.10			

CON = Control (without inoculant); LB = *Lactobacillus buchneri* CNCM I-4323 (10⁵ cfu/g of fresh forage); LP = *Lactobacillus plantarum* N2072 (10⁵ cfu/g of fresh forage).

¹GV = gas volume produced in liters per ton of dry matter; TDML = total DM losses; Gloss = gas losses; ELoss = effluent losses, kg per ton of fresh material.

²Standard error of mean. Capital letter (A; B) are the period comparison within the treatment (column); for the period comparison the T-test was used.

³P-value was considered significant when ≤ 0.05; treatment = inoculants, period = ensiling period, and treatment × period = interactions between treatment and period.

Table 6. Effects of *Lactobacillus* inoculants on microbial counts and aerobic stability (AS) test of total mixed ration (TMR) silages

Items	Ensiling period, days	CON	LB	LP	SEM ⁴	P-value ⁵		
						Treatment	Period	Treatment × Period
LAB, log cfu/g ¹	15	6.8	6.8	6.9	0.03	0.47	<0.01	0.73
	60	8.3	8.3	8.3	0.01			
Yeast, log cfu/g	15	4.3	4.1	3.9	0.11	0.66	<0.01	0.10
	60	3.2	3.2	3.3	0.05			
Molds, log cfu/g	15	2.6	3.0	3.2	0.22	0.71	0.39	0.91
	60	2.5	2.6	2.7	0.35			
AS ²	15	64.4 ^{Ba}	67.6 ^{Ba}	50.0 ^{Bb}	2.96	<0.01	<0.01	<0.01
	60	216 ^{Aa}	216 ^{Aa}	216 ^{Aa}	-			
TDMLas, % ³	15	18 ^{Ab}	18 ^{Ab}	23 ^{Aa}	8.08	<0.05	<0.01	<0.03
	60	1.2 ^{Ba}	1.1 ^{Ba}	0.5 ^{Ba}	1.56			

CON = Control (without inoculant); LB = *Lactobacillus buchneri* CNCM I-4323 (10⁵ cfu/g fresh forage); LP = *Lactobacillus plantarum* N2072 (10⁵ cfu/g fresh forage).

¹Lactic acid bacteria.

²Aerobic stability (hours for silage to increase temperature in 2 °C above room temperature).

³Total dry matter losses during aerobic stability trial.

⁴Standard error of mean; capital letter (A; B) are the period comparison within the treatment (column); for the period comparison the T-test was used. Lowercase letters (a; b) are the comparison of the treatments within the period in line; the Tukey test was used for treatment comparisons

⁵P-value was considered significant when ≤ 0.05; treatment = inoculants, period = ensiling period, and treatment × period = interactions between treatment and period.

DISCUSSION

According to McDonald et al. (1991), a small (5 to 6%) DM loss during the fermentation process in forage silages is normal; however, for the TMR silages, this loss appears to be less. Miyaji et al. (2013) evaluated the effect of replacing integral rice grains with maize grains in TMR silage and found a 0.7% DM loss when TMR was ensiled for 90 d. Chen et al. (2015) evaluated the effects of replacing the whole plant of oats by common oats and

vetch on the fermentation quality, chemical composition, and aerobic stability of TMR silage and found a 0.4% DM loss when the TMR silage was ensiled for 45 d.

A possible explanation as to why nutrient composition remained close to the initial values (Table 1) without being influenced by the inoculants, ensiling periods or interactions (Table 3), could be related to improved ensiling conditions in a laboratory setting, as well as high LAB population,

Figure 1. Effects of *Lactobacillus* inoculants on temperature (A and B) and pH (C and D) of total mixed ration (TMR) silage exposed to air after opening.

high DM content, and great bulk density of TMR silages. These results may vary in field conditions.

Microbial inoculants LP and LB decreased TMR silages NDF at 15 d of ensiling period (Table 3). This could be attributed to the occurrence of acid hydrolysis in the potentially digestible fractions of hemicellulose, notably from the nonfermented ingredients such as corn gluten meal and soybean meal, due to the reduced pH at the beginning of the ensiling process. However, the pH was similar among treatments at 15 d. Small proportions of hemicellulose can undergo chemical decomposition during silage fermentation and lactic acid bacteria can ferment sugars released by the decomposition of hemicellulose (McDonald et al. 1991). Thus, the decrease in NDF content due to inoculants at 15 d is probably related to hydrolysis factors other than the acids evaluated here. Muck et al. (2018) point out that LAB strains are capable of improving NDF digestibility by unclear mechanisms. Another possible explanation would be that greater DM loss in CON may have led to greater NDF concentration.

The increase of NH₃-N in the silage during the fermentation process is a usual indicative of poor silage quality due to *Clostridium* metabolism. This can occur by the deviation of the route of lactic fermentation, promoting proteolysis, turning proteins

and amino acids into harmful products to animal health, and causing higher losses (McDonald et al., 1991). The 15-d ensiling period for the TMR silage showed lower values of NH₃-N (16.0%) compared to 60 d (16.5%). The inoculants LB and LP had no effects on the NH₃-N concentration. Considering NH₃-N was already high at ensiling (15.6%), the initial pH was low (4.8), and the DM content was above 40% (Table 2), the increase in NH₃-N levels observed in the present study may be due to the presence of urea and soybean meal in these TMR (Table 1). The low content of butyric acid (Table 4) confirms this hypothesis. Lee et al. (2011) evaluated the effects of the addition of mixed microorganisms (molds, bacteria, and yeasts) on the fermentation of TMR and did not observe differences on NH₃-N due to treatments but observed a nonsignificant increase from the 1st to the 21st day of storage, which is similar to the results in the current experiment. According to Liu et al. (2016), NH₃-N concentrations below 10% of DM is an indicative of well-preserved TMR silages when no urea is added.

Silage pH is an important indicator to evaluate silage fermentation quality (Muck, 2013), but it depends on silage DM, ingredients, and ensiling periods. For TMR silages with high DM content

(greater than 40%; [Chen et al., 2015](#)), normal pH can range from 4.0 to 5.0. According to [Liu et al. \(2011\)](#), pH around 5.0 is acceptable when silage is ensiled with high DM content. [Nishino et al. \(2007\)](#) evaluated the fermentation process and aerobic stability of TMR silages inoculated with the *Lactobacillus casei* at 3×10^6 cfu/g fresh forage and LB at 1×10^6 cfu/g fresh forage and found that the inoculants could decrease the pH to 4.09 and 4.18, respectively, when the TMR silage was ensiled for 60 d.

During the silage fermentation process, organic acids, alcohols, aldehydes, esters, and ketones are produced ([Weiß et al., 2009](#)). This information may indicate if the silage fermentation process was adequate or not. [Liu et al. \(2016\)](#), evaluating the effects of inoculants on fermentation quality of TMR silage, ensiled for 60 d and found the greatest concentrations of lactic acid (3.8% DM) when LP plus fibrolytic enzymes were used. These concentrations of lactic acid in TMR silage are similar to those found in our study with the 60 d of ensiling (3.7% DM). [Nishino et al. \(2004\)](#) found concentrations of acetic acid and ethanol of 2.7 and 0.4% DM, respectively, for TMR ensiled for 10 d. When the silos were opened at 60 d, these values increased to 5.3% of acetic acid and 0.7% of ethanol. Similar responses were found in our study; at 15 d, TMR silage had the lower acetic acid and ethanol concentrations (2.4 and 0.4% DM, respectively); however, at 60 d, these values increased (2.7 and 0.6% DM).

[Wang et al. \(2010\)](#) found propionic acid concentrations of 0.04, 0.1, and 0.2% DM when the TMR was ensiled for 0, 7, and 14 d, respectively. These propionic acid values were lower than what we found in our study (0.3% DM), maybe because of the different ingredients in the TMR, DM content, and ensiling periods. [Chen et al. \(2015\)](#), evaluating the effects of replacement of whole-plant corn with oat and common vetch on TMR silage fermentation quality, ensiled for 45 d found a butyric acid concentration of 0.02% DM for the control treatment and when the oat and common vetch were mixed with the control, butyric acid was not found. The same pattern was found in our study, when TMR was ensiled for 15 d the control treatment had the greatest butyric acid concentration (0.03% DM), showing that the LB and LP inoculants were efficient in decreasing the concentration of butyric acid at 15 d (0.02% DM). However, at 60 d, the 3 treatments show the same concentration of butyric acid (0.02% DM).

During TMR silage fermentation period, lactic acid is the most abundant final product produced

by LAB. This process can preserve almost all DM and energy. Acetic acid is also desirable if it is achieved via controlled fermentation, such as LB, as it inhibits the proliferation of yeast and mold, which increases aerobic stability after silo opening. However, [Muck \(2010\)](#) reported that the production of acetic acid through obligate heterofermentative LAB results in additional loss of CO₂ and reduces DM recovery by approximately 1 percentage point on average; however, in our study, there was no increase in acetic acid concentration and no decrease in DM recovery in silages treated with LB. The high DM content of TMR silage may have inhibited the metabolism of LB, which can lead to a small increase in acetic acid concentration ([Table 4](#)). [Driehuis et al. \(2001\)](#) found increased effect of LB inoculation with decreasing of DM content of perennial ryegrass silages, from 42 to 24% of DM.

The conversion of soluble carbohydrates into ethanol results in high DM losses ([McDonald et al., 1991](#); [Bolsen et al., 1992](#)), however, when consumed by animals, ethanol can be absorbed or metabolized by rumen microorganisms ([Kristensen et al., 2007](#)), becoming a high energy compound. According to [Wang and Nishino \(2008\)](#), crops with high contents of DM and high sugar content sometimes produce more alcoholic silages; so, the TMR silage may be more prone to alcoholic fermentation than conventional silage. However, in the present study, the average values of ethanol can be considered low, even for silages of RMT stored for 60 d (0.6% of DM). No effect of LB over ethanol was detected, probably due to the limited action of this inoculant over yeasts, in the condition of the present study.

The gas volume and fermentation losses (TDML, Gloss, and ELoss) of the TMR silage were low ([Table 5](#)). [Tabacco et al. \(2011\)](#) evaluated the effects of the microbial inoculants LB and LP on fermentation losses in corn and sorghum silages and found 2.4 to 3.4% DM loss for corn silage and 2.0 to 2.8% DM loss for sorghum silage when silages were ensiled for 90 d. [Oliveira et al. \(2010\)](#) evaluated the fermentation losses of corn silage, sudan sorghum silage, forage sorghum silage, and sunflower silage at 60 d and found gas losses of 2.2, 5.3, 7.4, and 2.2% and effluent losses of 20, 97, 70, and 38 kg/t fresh forage, respectively.

Comparing the fermentation loss values of TMR silages with those found in these studies, the TMR silages had lower fermentation losses, which may be related to the efficiency of compaction, adequate sealing, and high DM content. However, LB and LP inoculants did not reduce the fermentation losses of the TMR silage at 15 or 60 d.

Although gas volume produced (GV) was lower at 15 d, the gravimetric values to estimate TDML and GLoss were lower for silos opened after 60 d (Table 5). This may be due to using oven-dried DM values to calculate loss estimates. The greater lactic acid content in TMR opened at 60 d may have led to lower organic compounds disappearance in the oven, reducing the estimates of losses. This effect is consistent with the limitation of gravimetric estimates of losses based on oven-dried samples, without correction for volatile compound losses.

The microorganism population in silage depends on several factors and quantifying them is important as they may reveal whether there was any undesirable microorganisms' activity during the fermentation process (Muck, 2010). Lactic acid bacteria mainly produce lactic acid that can help decrease the pH of the ensiled material. The faster this happens during the fermentation process, the lower are the fermentation losses in silage (Muck, 2013).

In the TMR silages, LB and LP inoculants did not change LAB, yeast, and mold counts; however, the 15 d ensiling period had a lower LAB population, greater counts of yeasts, and greater DM loss when the material was exposed to air (TDMLas). Hu et al. (2015) evaluated the LAB population of TMR silages stored from 1 to 56 d of ensiling. Authors found the greatest LAB counts at 14 d (8.4 log cfu/g) which decreased to 6.6 log cfu/g at 56 d. *Pediococcus acidilactici*, *L. brevis*, *L. plantarum*, and *P. pentosaceus* were the main LAB at 14 d, while *P. acidilactici* and *L. buchneri* were dominant at 56 d. The high soluble carbohydrate concentration available in peach pomace used in these TMR may explain the fast growing of LAB in these silages. So, the ensiling period together with the silage ingredients can influence the LAB count and, in our study, the 60-d period had the greatest microbial count.

The decrease in yeast counts in TMR silage was also found by Hu et al. (2015), with values of 10^6 cfu/g of fresh silage when ensiled for 14 d, decreasing to 10^4 cfu/g of fresh forage ensiled for 56 d. Wang and Nishino (2013), evaluated the influence of temperature (5, 15, 25, and 35 °C) and the ensiling period (10, 30, and 90 d) on fermentation process, aerobic stability, and microbial population of TMR silage and found similar responses to the present study. When the TMR was ensiled longer, lower yeast counts were observed. Based on the results of the present study, we can affirm that changes occur in the microbial population of the TMR silages during the ensiling period. When LAB

increases, and yeast and mold decrease, it means that the silage fermentation process was appropriate, and the losses will likely be small. In our study, the 15-d ensiling period appears to be insufficient for adequate microbial fermentation.

Prolonging the time for silage aerobic stability breakdown has received considerable interest lately (Nishino et al., 2004; Nishino and Hattori, 2007; Yuan et al., 2015). According to Wang and Nishino (2008), the aerobic deterioration of TMR silage with forage sorghum hay and alfalfa hay occurs when the material is ensiled for short periods (14 d), corroborating with the results observed in our study. Wang and Nishino (2009) evaluated the effects of LB on the aerobic stability of TMR silage containing moist brewery residues and concluded that TMR is a good option for preserving wet residues in the form of silage and the additive LB played an important role on inhibiting aerobic deterioration.

When all steps of making TMR silage are well performed, it can be created in an environment that is adequate for the development of LAB and consequently can decrease yeast and mold counts and then increase the number of hours for breaking down the aerobic stability (Nishino et al., 2004; Hu et al., 2015). Therefore, the use of microbial inoculants such as LB and LP in TMR silage is dependent on the ingredients that compose the silage, the LAB initial population, the applied rate, and the time that the material will remain ensiled.

In general, under the conditions of our study, the microbial inoculants LB and LP were not efficient in improving the fermentation process of TMR silage and the concentrations of organic acids, alcohols, aldehydes, esters, and ketones are mainly dependent on the microbial population, DM content, storage period, and the ingredients that make up the TMR.

CONCLUSIONS

Under the experimental conditions of the present study, the inoculants LB and LP did not improve fermentation profile, did not prolong aerobic stability, and did not reduce TMR silage losses. However, TMR had desirable characteristics to be stored as silage and a 60-d ensiling period was sufficient to allow adequate bacterial activity. Therefore, based on these results, we do not recommend the use of these inoculants at the levels tested and we recommend ensiling periods of at least 60 d for TMR diets.

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