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Lactobacillus buchneri inoculation compared to chitosan and facultative heterofermentative lactic acid bacteria improves sugarcane silage conservation

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Abstract

The present study aimed to evaluate the effects of chitosan instead of microbial inoculants on fermentation profile, losses, chemical composition, *in vitro* degradation, and aerobic stability of sugarcane silage (SS). Forty experimental silos (PVC tubes with 28 cm i.d., 25 cm height) were used in a randomized block design to evaluate the following treatments: (I) Control (CON): SS with no additive; (II) LB: SS ensiled with 5.0×10^5 colony forming units (CFU) of *Lactobacillus buchneri* (NCIM 40788)/g as-fed; (III) LPPA: SS ensiled with 1.6×10^5 CFU of *L. plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g as-fed; and (IV) Chitosan (CHI): SS ensiled with 6 g/kg dry matter (DM) of chitosan. Microbial inoculation of SS reduced ($P \leq 0.05$) silage pH relative to CON and CHI treatment. The LPPA decreased ammonia-nitrogen and LB decreased ($P \leq 0.05$) ethanol content and increased acetic acid content relative to other treatments. The LPPA-silos had higher ($P \leq 0.05$) gas losses and lower ($P \leq 0.05$) DM recovery than other treatment silos. Consequently, LPPA reduced ($P \leq 0.05$) DM and non-fibre carbohydrates and increased ($P \leq 0.05$) neutral detergent fibre (NDF) silage content compared to other treatments. Treatments did not affect ($P \geq 0.212$) DM and NDF *in vitro* degradation and silage pH after aerobic exposure. However, LB reduced silage temperature after aerobic exposure. Thus, LB reduces alcoholic fermentation and improves SS aerobic stability. Inoculation of LPPA reduces DM recovery and negatively affects SS chemical composition. Although CHI positively affects SS conservation relative to CON, it shows higher gas losses and decreased SS temperature after aerobic exposure compared to LB.

Introduction

Sugarcane (*Saccharum* spp.) silage can be a forage source for ruminants since the harvesting phase coincides with the winter, a period of pasture shortage (Bernardes and do Rêgo, 2014; Oliveira and Millen, 2014). The sugarcane silage (SS) has high biomass productively (70,357 kg of natural matter/ha – CONAB, 2022) with low production cost. However, alcoholic fermentation by yeasts could comprise SS ensiling. This fermentation increases gas losses, resulting in low-quality and decreased dry matter (DM) recovery (Pedroso *et al.*, 2005; Daniel *et al.*, 2015).

The limitations of natural fermentation in SS led to the identification of additives that inhibit epiphytic yeast populations and mitigate nutrient losses (Ávila *et al.*, 2014). Additives such as lactic acid bacteria (LAB) increase the quality of forage fermentation (Kleinschmit and Kung, 2006; Rabelo *et al.*, 2019). The LAB regulate yeast growth by producing high concentrations of organic fatty acids, such as acetic and propionic acids, which have a fungicidal effect (Moon, 1983). Consequently, LAB decrease ethanol production and improve silages' aerobic stability (Driehuis *et al.*, 1999; Kleinschmit and Kung, 2006).

Obligatory heterofermentative (i.e. *L. buchneri*) and facultative heterofermentative (i.e. *Lactobacillus plantarum* and *Pediococcus acidilactici*), and have been used as silage additives (Ávila *et al.*, 2014; Carvalho *et al.*, 2014; dos Santos *et al.*, 2015). *Lactobacillus plantarum* and *P. acidilactici* were evaluated on SS without benefits to justify their use, but studies are limited and hence more studies have to be carried out (Carvalho *et al.*, 2014; dos Santos *et al.*, 2015). *Lactobacillus buchneri* reduces DM losses and increases the aerobic stability of SS (Pedroso *et al.*, 2008; Ávila *et al.*, 2009).

New additives are being used in SS, improving the fermentation process. Among these additives, we could mention chitosan (CHI) (Gandra *et al.*, 2016; Del Valle *et al.*, 2020). Chitosan is a biopolymer obtained by partial deacetylation of chitin. Its antimicrobial activity

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had been well documented (Şenel and McClure, 2004; Deng *et al.*, 2020). Del Valle *et al.* (2018) reported that 6 g of CHI/kg DM reduced fermentative losses, besides reduced fibre content and increased DM degradation of SS. Gandra *et al.* (2016) used 10 g of CHI/kg as-fed (36 g/kg DM), finding a higher DM content, fewer total losses, and higher neutral detergent fibre (NDF) *in vitro* degradation. Del Valle *et al.* (2020) reported that CHI linearly increased acetic acid concentration, reduced ethanol production, and improved silage DM recovery.

We hypothesized that microbial inoculants and CHI would positively affect the fermentation pattern and aerobic stability, improving DM recovery, nutritional value, and DM and NDF *in vitro* degradation of SS. The objective of the present study was to evaluate the effects of two different microbial inoculants and CHI on the parameters of SS, such as fermentation profile, losses, chemical composition, *in vitro* degradation, and aerobic stability.

Materials and methods

The trial was performed in the Group of Agricultural Studies and Work (GETAP) of Agrarian Sciences Center (CCA), Federal University of São Carlos (UFSCar), from July to October 2019. The Ethics Committee of UFSCar previously approved experimental procedures (approval number: 1,395,120,219).

Experimental design and treatments

Forty experimental silos (PVC tubes with 28 cm inside diameter, 25 cm height) were used in a randomized block design to evaluate the following treatments: (I) Control (CON): SS ensiled with no additive; (II) LB: SS ensiled with 5.0×10^5 colony forming units (CFU) of *L. buchneri* (NCIM 40788, Lasil Cana®, Lallemand Animal Nutrition, Montreal, Canada)/g of fresh matter; (III) LPPA: SS ensiled with 1.6×10^5 CFU of *L. plantarum* and 1.6×10^5 CFU of *P. acidilactici* (Kera SIL®, Kera Nutrição Animal, Bento Gonçalves, Brazil)/g of fresh matter; and (IV) Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan. *Lactobacillus buchneri* dose was defined according to the manufacturer and was slightly higher than recommended by Kleinschmit and Kung (2006) to promote aerobic stability (4×10^5 CFU/g of fresh matter). *Lactobacillus plantarum* and *P. acidilactici* doses were defined according to the manufacturer's suggestion. Chitosan level was defined based on Del Valle *et al.* (2020). Chitosan (Polymar, Fortaleza, Brazil) used in the present study showed 0.640 density, 883 g/kg DM, 20.0 g/kg of ash, and 7.0 to 9.0 pH.

Ensiling, sampling, and data record

Five sugarcane cultivars (RB 985476, RB 975201, RB 975952, RB 935744, and RB 975242) plots were harvested at 5-cm height to produce eight silos (2 per treatment) from every cultivar. Plants were chopped in a stationary hammer mill (TRF300®, Trapp, Jaguará do Sul, Brazil). One sugarcane sample of each cultivar (Table 1; $n = 5$) was collected for chemical analysis and degradation assay. Brix was determined according to the refractometric method (# 990.35, AOAC, 2000). Cultivars were defined as blocks to allow inference in the sugarcane population (Kononoff, 2020). Two experimental units from the same block in each treatment were defined by the ensiling period (morning and afternoon).

Silos were prepared with 5 kg of oven-dried sand (80°C for 24 h) and a nylon screen in the bottom to allow effluent quantification and avoid air contamination. The sugarcane of each silo was weighed (7.80 kg) to find 650 kg/m^3 of density. Individual treatment was applied and manually mixed to sugarcane. Distilled water (50 ml per silo) was used to permit spray to apply of microbial inoculants. Control and CHI-silos received the same water volume to obtain the placebo effect. Silos were weighed, before and after fill and sealing, using a 5-g sensitivity scale (Mettler Toledo, Barueri, Brazil). Silos were stored for 90 days at room temperature, without sun and rain contact. The ambient temperature averaged 22.0°C (ranging from 9.9 to 36.6°C) during the storage period. Silos' weight was recorded at 15, 25, 35, 45, 55, 65, 74, and 90 days after the ensiling to evaluate gas losses through the ensiling process. After the storage period, silos were opened. The topmost and the bottom layers (5-cm) were discarded, and silage was homogenized for sampling. One sample (~ 500 g) was used to obtain silage fluid using a hydraulic press (PHE-45®, Engehidro, Piracicaba, Brazil). Silage pH was immediately recorded using a benchtop potentiometer (LUCA210®, Lucadema, Sao José do Rio Preto, Brazil) and the fluid was frozen for fermentation profile evaluation. Another sample (~500 g) was collected, dried in a forced-air oven for 72 h at 60°C, and ground in a knives mill (SL-31, Solab Científica, Piracicaba, Brazil), using a 2-mm and 1-mm sieve for *in vitro* assay and chemical analysis, respectively. Thus, another sample (3 kg) was moved back to PVC tubes and stored for 144 h in a controlled temperature room (21.3 ± 1.24°C; mean ± SD) without compaction. Silage pH was recorded every 24 h after water solubilization [10 g in 100 ml of distilled water (Cherney and Cherney, 2003)], using a benchtop potentiometer, as previously described. The temperature was evaluated every 8 h using spit thermometers (K29-5030®, Kasvi Produtos Laboratoriais, Pinhais, Brazil). The aerobic stability period was defined as the time when the silos temperature remained up to 2°C higher than the environment (Ranjit and Kung, 2000).

Table 1. Chemical composition and *in vitro* degradation of sugarcane before the ensiling ($n = 5$; g/kg DM, unless stated)

Item	Mean	SD
Dry matter, g/kg natural matter	218	31.6
Organic matter	977	5.0
Neutral detergent fibre	535	74.6
Acid detergent fibre	474	70.8
Non-fibre carbohydrates	404	82.9
Crude protein	28.3	5.02
Ether extract	9.73	2.207
BRIX	209	32.2
In vitro degradation		
Dry matter	606	57.3
Neutral detergent fibre	358	178.2

Chemical analysis

Silage fluid was thawed at room temperature and centrifuged at $500 \times \boldsymbol{g}$ for 15 min. Ammonia-nitrogen (NH_3-N) was analysed using the Kjeldahl method (984.13; AOAC, 2000) without sample digestion. The fluid lactic acid concentration was analysed

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according to Pryce (1969), using the spectrophotometric method. Other organic acids (acetic, propionic, butyric, valeric, iso-valeric, and isobutyric acids) and ethanol concentrations were evaluated using the chromatographic method. The sample (1.8 ml) was acidified using ortho-phosphoric acid (0.2 ml). A gas chromatography (GC-2010 Plus chromatograph, Shimadzu, Barueri, Brazil) equipped with an auto-sampler (AOC-20i), a capillary column (Stabilwax-DA™, 30 m, 0.25 mm i.d., 0,25 µm df, Restek©), and a flame ionization detector was used. The sample (1 µl) was injected using a 40:1 split ratio, using He as carrier gas (42 m/s of linear velocity). Injector temperature was 250°C and detector temperature was 300°C. The following standards were used to calibrate the chromatographic method: WSFA-2 (Ref. 47056, Supelco©) and ethanol (Ref. 459828, Sigma-Aldrich©).

Chemical analyses were performed using 1-mm processed samples. It was analysed DM (method 950.15), crude protein (CP, N × 6.25, Kjeldahl method 984.13), ether extract (EE, method 920.39), and organic matter (OM = 1000- crude ash; method 942.05) according to AOAC (2000). Neutral detergent fibre and acid detergent fibre (ADF) were determined according to Van Soest *et al.* (1991). Non-fibre carbohydrate (NFC) was calculated as NFC = 1000 − (NDF + CP + EE + ash). Silage protein fractions were analysed using methods described by Licitra *et al.* (1996). It was accessed non-protein-N (A-fraction) using trichloroacetic acid, acid-detergent insoluble-N (fraction C), and neutral-detergent insoluble-N (NDIN). The B3 fraction was obtained by NDIN and C-fraction difference. The B1 plus B2 fractions were obtained by the difference between the total N and A fraction plus NDIN.

In vitro assay

DM and NDF *in vitro* degradation were analysed according to Holden (1999) technique. 2-mm processed samples were placed into non-woven fabric bags (100 g/m^2; Casali *et al.*, 2008). The internal dimension of the bags was 5 × 5 cm and the sample weight was set at less than 20 mg DM/cm^2 (Nocek, 1988). Bags (triplicates per sample) were incubated for 48 h at 39°C in an *in vitro* incubator (NL162®, New Lab, Piracicaba, Brazil). Each incubator vial received 2.0 l of ruminal inoculum [1.6 l of McDougall (1948) buffer and 0.4 l of fresh ruminal fluid]. Ruminal fluid was sampled from two Holstein heifers (500 kg of body weight) maintained in a *Megathyrsus maximus* pasture with no concentrate supplementation. After incubation, bags were washed in running tap water, dried at 55°C for 72 h and at 105°C for two hours to assess undigestible DM (Detmann *et al.*, 2012), and sample NDF content was analysed as previously described.

Calculations

Gas losses (GL; Eqn 1), effluent production (EP; Eqn 2), and DM recovery (DMR; Eqn 3) were analysed as follows (Jobim *et al.*, 2007):

$$\mathrm{GL}\left(\frac{g}{\text{kg DM}}\right) = \frac{[\mathrm{WSW}_{en}(g) - \mathrm{WSW}_{op}(g)]}{\text{EDM (kg)}} \quad (1)$$

where WSW is the whole silo weight, *en* and *op* stands for weight at ensiling and at opening, respectively; and EDM is the ensiled DM. Gas losses through the ensiling process were calculated using the same equation and considering every partial weight as WSW_{op}.

$$\mathrm{EP}\left(\frac{g}{\text{kg DM}}\right) = \frac{[\mathrm{ESW}_{op}(g) - \mathrm{ESW}_{en}(g)]}{\text{EDM (kg)}} \quad (2)$$

where ESW is the empty silo weight (containing sand). DM recovery was obtained by the ratio between opening DM (ODM) and ensiling DM, as following:

$$\mathrm{DMR} = \frac{\mathrm{ODM}}{\mathrm{EDM}} \quad (3)$$

Statistical analysis

Statistical analysis was performed using PROC MIXED of SAS (version 9.4, SAS Inst. Inc., Cary, NC, USA), and the following model:

$$Y_{ijk} = \mu + T_i + s_j + e_{ijk} \quad (4)$$

with

$s_j \approx N(0,\ \sigma_s^2)$ and $e_{ijk} \approx N(0,\ \sigma_e^2)$; where:
Y_{ijk} is the observed value of response variable ($n = 40$);
μ is the general mean;
T_i is the fixed effect of treatment ($i = 1$ to 4);
s_j is the random effect of sugarcane cultivar ($j = 1$ to 5); e_{ijk} is the random experimental error; N stands for Gaussian distribution;
σ_s^2 and
σ_e^2 are variances associated with sugarcane cultivar and experimental error, respectively.

Silage pH and temperature after aerobic exposure were evaluated as repeated measures, using the following model:

$$Y_{ijkl} = \mu + T_i + s_j + \omega_{ijk} + H_l + T \times H_{il} + e_{ijkl} \quad (5)$$

with $s_j \approx N(0,\ \sigma_s^2)$, $\omega_{ijk} \approx N(0,\ \sigma_\omega^2)$, and $e_{ijkl} \approx MVN(0,\ R)$ where: Y_{ijkl} is the observed value response variable; μ is the general mean; μ, T_i, s_j, N, and σ_s^2 were previously described; ω_{ijk} is the error associated with experimental units (silos); H_l is the fixed effect of time/hours after aerobic exposure ($l = 1$ to 18 for silage temperature; and 1 to 6 for silage pH); $T \times H_{il}$ is the fixed interaction between treatment and time and time effects; e_{ijkl} is the experimental error; σ_ω^2 is the variance associated with experimental units (silos) error; *MVN* stands for multivariance normal distribution; R is a variance and covariance matrix due to repeated measures. Matrix (CS, CSH, AR, ARH, TOEP, TOEPH, FA, UN, ANTE) were evaluated using the Bayesian method. For all analyses, additives effects were decomposed using the Fisher's protected means test. Significance was declared at $P \leq 0.05$.

Results

Fermentative profile

Microbial inoculation of SS reduced ($P \leq 0.05$) silage pH relative to CON and CHI treatment (Table 2). Additionally, LPPA inoculation decreased ($P \leq 0.05$) NH_3-N and LB treatment decreased ($P \leq 0.05$)

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Table 2. Silage fermentation profile of sugarcane silage treated with microbial inoculants and chitosan

Item	Treatments[1]				S.E.M.[2]	P^3
	CON	LB	LPPA	CHI		
pH	3.81[a]	3.66[b]	3.72[b]	3.78[a]	0.012	< 0.001
NH_3-N, g/kg N	215[a]	234[a]	188[b]	220[a]	18.1	0.008
Ethanol, mg/kg DM	130[b]	92.7[c]	164[a]	137[ab]	8.2	< 0.001
Organic acids, g/kg DM						
Lactic acid	37.6[b]	36.1[b]	51.2[a]	45.5[a]	1.60	0.002
Acetic acid	25.8[b]	55.5[a]	22.5[b]	20.6[b]	2.57	< 0.001
Butyric acid, mg/kg DM	608	593	783	943	199.6	0.250
BCFA[4], mg/kg DM	104[a]	86.4[ab]	82.6[ab]	59.0[b]	5.70	0.037
Propionic acid, mg/kg DM	35.5	47.0	43.1	33.8	4.14	0.464
Lactic to acetic acid ratio	1.58[bc]	0.858[c]	3.22[a]	2.29[ab]	0.186	0.001

[a–c]Fisher means test (LSD) at 5% of probability.
[1]Control (CON): SS with no additive; LB: SS with 5.0×10^5 CFU of *Lactobacillus buchneri* (NCIM 40788)/g of fresh matter; LPPA: SS with 1.6×10^5 CFU of *Lactobacillus plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g of fresh matter; and Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan.
[2]Standard error of mean;
[3]Probability of treatment effect;
[4]Branched-chain fatty acids.

ethanol concentration in relation to other treatments. Chitosan and LPPA-treated silos showed higher contents than CON and LB-treated ones, whereas LB increased ($P \leq 0.05$) acetic acid silage concentration compared to other treatments. Therefore, the lowest lactic to acetic concentration was observed in LB silos, and the highest ratio was observed in LPPA-silos. Additionally, CHI showed lower ($P \leq 0.05$) branched-chain fatty acids (BCFA) concentration than CON.

Fermentative losses, DM recovery, and chemical composition

There was no treatment effect ($P = 0.571$) on effluent losses (Table 3). However, LPPA-silos had higher ($P \leq 0.05$) gas losses (Fig. 1) and lower ($P \leq 0.05$) DM recovery than silos of other treatments. LB reduced ($P \leq 0.05$) gas losses compared to CHI, but both treatments had similar ($P > 0.05$) DM recovery. The LPPA-treatment reduced ($P \leq 0.05$) DM and NFC, whereas

Table 3. Fermentative losses, dry matter recovery, and chemical composition of sugarcane silage treated with microbial inoculants and chitosan (g/kg DM, unless stated)

Item	Treatments[1]				S.E.M.[2]	P^3
	CON	LB	LPPA	CHI		
Fermentative losses						
Effluent losses, g/kg NM[4]	124	128	129	126	6.6	0.571
Gas losses	197[bc]	186[c]	226[a]	202[b]	11.4	< 0.001
Dry matter recovery, g/kg	751[a]	766[a]	688[b]	748[a]	33.3	0.003
Chemical composition						
Dry matter, g/kg NM[4]	192[a]	195[a]	178[b]	192[a]	2.2	0.009
Organic matter	972	973	971	972	1.0	0.850
Neutral detergent fibre	706[b]	718[b]	749[a]	706[b]	10.1	0.033
Acid detergent fibre	439[ab]	425[b]	463[a]	439[ab]	10.3	0.068
Non-fibre carbohydrates	209[a]	203[a]	148[b]	208[a]	11.3	0.009
Crude protein	28.0	27.9	28.5	28.0	0.77	0.953
Ether extract	29.0[ab]	23.6[b]	36.0[a]	29.3[ab]	1.8	0.109

[a–b]Fisher means test (LSD) at 5% of probability.
[1]Control (CON): SS with no additive; LB: SS with 5.0×10^5 CFU of *Lactobacillus buchneri* (NCIM 40788)/g of fresh matter; LPPA: SS with 1.6×10^5 CFU of *Lactobacillus plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g of fresh matter; and Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan.
[2]Standard error of mean.
[3]Probability of treatment effect.
[4]Natural matter.

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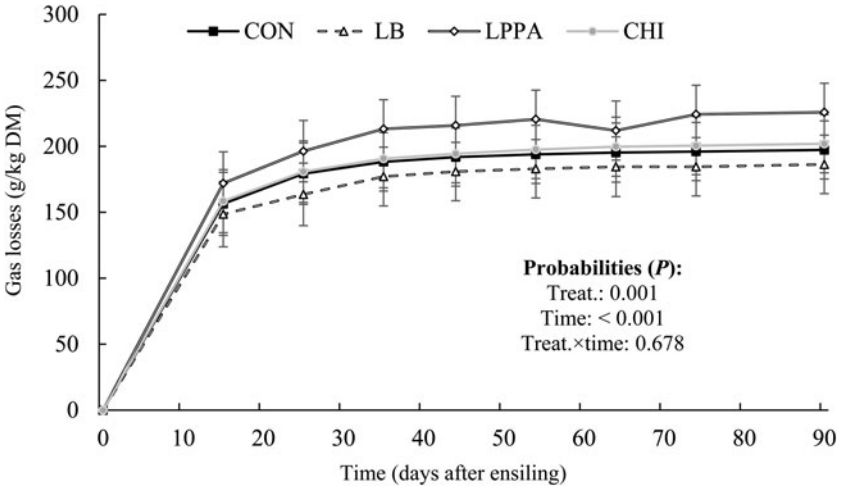


Fig. 1. Gas losses through the ensiling process of sugarcane silage treated with microbial inoculants and chitosan. [1]Control (CON): SS with no additive; LB: SS with 5.0×10^5 CFU of *Lactobacillus buchneri* (NCIM 40788)/g of fresh matter; LPPA: SS with 1.6×10^5 CFU of *Lactobacillus plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g of fresh matter; and Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan. [2]Standard error of mean; [3]Probability of treatment effect.

increased ($P \leq 0.05$) NDF silage concentration compared to other treatments. Evaluating microbial inoculated silos, LB decreased ($P \leq 0.05$) ADF and EE silage content relative to LPPA.

In vitro degradation and protein fractions

There were no treatment effects ($P \geq 0.212$) on *in vitro* degradation of DM and NDF (Table 4). In addition, treatments did not affect ($P \geq 0.075$) B1 + B2, B3, and C fraction of protein. However, CON had a higher ($P \leq 0.05$) A protein fraction than other treatments, and CHI showed a lower ($P \leq 0.05$) A protein fraction compared to LB.

Aerobic stability

Treatments had no effect ($P = 0.344$) on silage pH after aerobic stability, regardless ($P = 0.363$) of the time of evaluation (Fig. 2). Similarly, treatments did not affect ($P = 0.545$) the period of aerobic stability (Table 4), and there was no treatments and time interaction effect ($P = 0.790$) on temperature after aerobic exposure. However, in general, LB decreased ($P \leq 0.05$) silage temperature compared to CON and LPPA treatments (Fig. 3). Besides, CON-silos showed higher ($P \leq 0.05$) temperatures after aerobic exposure than CHI-silos.

Discussion

As hypothesized, LB promoted heterolactic fermentation, reduced fermentation losses, and decreased silage temperature of SS after aerobic exposure. Although chitosan also had decreased temperature after aerobic exposure and improved DM recovery compared to LPPA, it had lower effects than LB on the SS fermentation profile. LPPA-inoculation increased gas losses and comprised DM recovery and carbohydrates composition compared to control.

In the present study, SS inoculation (LB and LPPA) reduced silage pH compared to CON and CHI treatments. Reduced silage pH in LPPA-silages occurred because homofermentative (or facultative heterofermentative) LAB promote lactic acid production from water-soluble carbohydrates and reduce silage pH (Ávila

Table 4. *In vitro* degradation, protein fractions, and time of aerobic stability of sugarcane silage treated with microbial inoculants and chitosan

	Treatments[1]					
Item	CON	LB	LPPA	CHI	S.E.M.[2]	*P*[3]
In vitro degradation, g/kg						
Dry matter	472	506	476	485	14.2	0.519
Neutral detergent fibre	251	311	298	268	20.8	0.212
Protein fractions[4], g/kg N						
A	343[a]	285[b]	244[bc]	221[c]	19.0	< 0.001
B1 + B2	295	335	383	371	18.8	0.075
B3	56.8	86.6	64.8	104	11.4	0.234
C	305	294	308	324	12.1	0.588
Aerobic stability, h	36.1	48.8	37.7	38.8	4.20	0.545

[a–b]Fisher means test (LSD) at 5% of probability.
[1]Control (CON): SS with no additive; LB: SS with 5.0×10^5 CFU of *Lactobacillus buchneri* (NCIM 40788)/g of fresh matter; LPPA: SS with 1.6×10^5 CFU of *Lactobacillus plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g of fresh matter; and Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan.
[2]Standard error of mean.
[3]Probability of treatment effect.
[4]Protein fractions: A: non-protein nitrogen; B: available protein, where B3 is neutral-detergent insoluble N and B1 + B2 is neutral detergent soluble N; C is unavailable protein.

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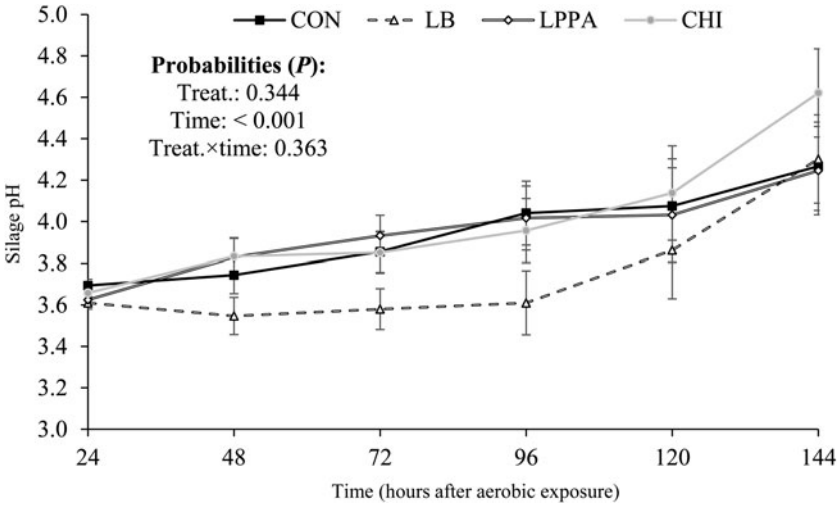


Fig. 2. pH after aerobic exposure of sugarcane silage treated with microbial inoculants and chitosan. [a–b]Fisher means test (LSD) at 5% of probability. [1]Control (CON): SS with no additive; LB: SS with 5.0×10^5 CFU of *Lactobacillus buchneri* (NCIM 40788)/g of fresh matter; LPPA: SS with 1.6×10^5 CFU of *Lactobacillus plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g of fresh matter; and Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan. [2]Standard error of mean; [3]Probability of treatment effect.

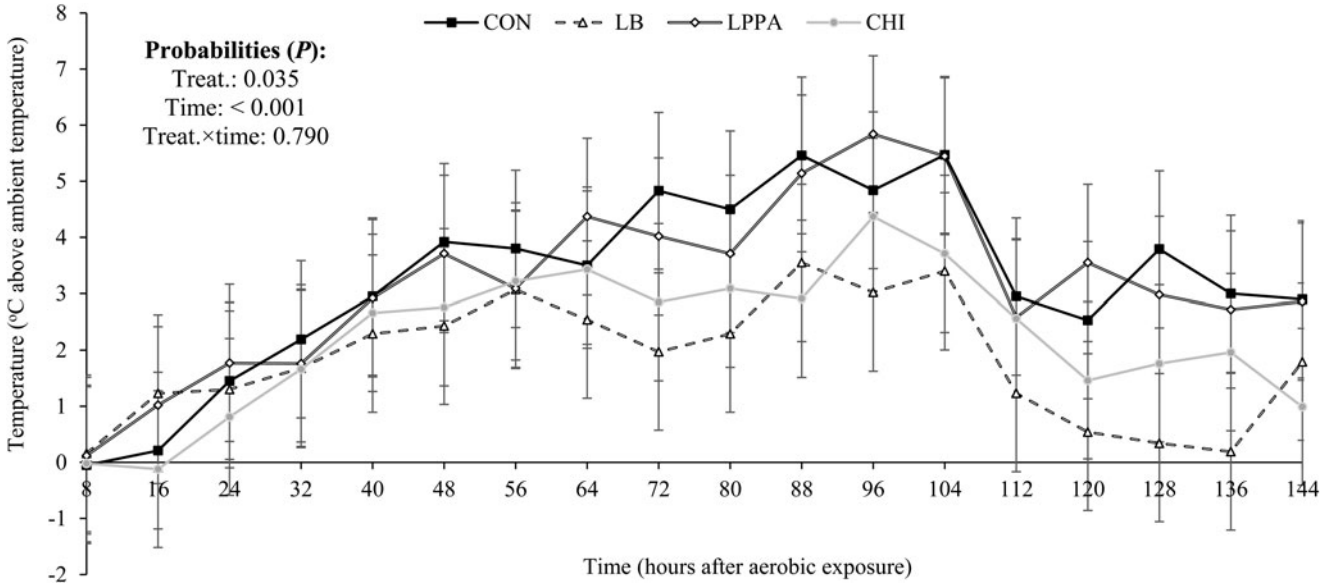


Fig. 3. Temperature after aerobic exposure of sugarcane silage treated with microbial inoculants and chitosan. Means: CON: 3.37[a]; LB: 1.95[c]; LPPA: 3.24[ab]; and CHI: 2.26[bc]. [a–b]Fisher means test (LSD) at 5% of probability. [1]Control (CON): SS with no additive; LB: SS with 5.0×10^5 CFU of *Lactobacillus buchneri* (NCIM 40788)/g of fresh matter; LPPA: SS with 1.6×10^5 CFU of *Lactobacillus plantarum* and 1.6×10^5 CFU of *Pediococcus acidilactici*/g of fresh matter; and Chitosan (CHI): SS ensiled with 6 g/kg DM of chitosan. [2]Standard error of mean; [3]Probability of treatment effect.

et al., 2014; dos Santos *et al.*, 2015), once lactic acid has lower pKa than other organic acids (Moon, 1983; Gandra *et al.*, 2016). Reduced silage pH observed in LB inoculated silages is a non-expected result. Arriola *et al.* (2021) reported that a high inoculation rate of LB could reduce silage pH. However, the exact action mechanism is not clear. Previous studies observed an increase in the pH of SS when chitosan was added during the ensiling (Gandra *et al.*, 2016; Del Valle *et al.*, 2018). This effect had been associated with the antimicrobial effect of chitosan and the absence of chitosan effect on silage pH seems associated with slight effects observed on other evaluated variables.

The use of LB reduced the lactic to acetic acid ratio compared to other treatments. LB-inoculated silages typically contain more acetate and less lactate than untreated silages because LB converts lactate to acetate and 1,2-propanediol during silage fermentation (Oude-Elferink *et al.*, 2001). Metanalytic studies have reported 30.8% (Rabelo *et al.*, 2019) and 61.8% (Arriola *et al.*, 2021) positive effects of LB treatment on acetic acid silage concentration. In the present study, LB increased (29.7 g/kg or 115%) acetic acid concentration, compared to CON treatment. On the other hand, LPPA and CHI had higher lactic acid concentrations relative to other treatments in the current study. The increased lactic acid concentration in LPPA-silages is a consequence of the lower production of other organic acids during the fermentation process (Oliveira *et al.*, 2017). Increased lactic acid concentration observed in CHI-treated silages has not been observed in previous studies of our research group (Del Valle *et al.*, 2018, 2020; de Morais *et al.*, 2021). Only Gandra *et al.* (2016) observed increased lactic acid concentration in CHI-treated silage. This effect was associated with increased LAB count.

Acetic acid has an antifungal effect (Moon, 1983) and can decrease yeast activity after aerobic exposure (Kleinschmit and

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Kung, 2006). This effect seems to reflect in numerical lower silage pH and lower temperature observed in LB-silos compared to CON and LPPA-silos, regardless of evaluated time. Arriola *et al.* (2021) reported a 73.8% increase in the time of aerobic stability using LB inoculation. However, there was no treatment effect on the aerobic stability period, in the present study. The LB had 12.7 h (35.2%) more aerobic stability, and LB significantly decreased average temperature after aerobic exposure relative to CON. In addition, no difference was observed between CHI and LB in silage pH and temperature after aerobic exposure. The positive effect of chitosan on aerobic stability has been associated with direct inhibition of yeast and mould after air exposure (Del Valle *et al.*, 2018, 2020). Chitosan can suppress fungal sporulation and spore germination (Hernandez-Lauzardo *et al.*, 2011), especially in acid environmental conditions.

Lactobacillus plantarum increase by 43.8% the concentration of ethanol in SS, resulting in 9.6% increased DM losses (Rabelo *et al.*, 2019). In our experiment, LPPA reduced DM recovery and increased gas losses compared to other treatments, similar to Rabelo *et al.* (2018). The LPPA-silos showed + 26.2%, + 14.7%, and −8.39% effects on ethanol concentration, gas losses, and DM recovery, respectively. As LPPA increased fermentation losses, it reduced DM and increased NDF silage content compared to other treatments. These results can be explained by excessive growth of yeast under anaerobic conditions, leading to DM losses due to two moles of ethanol and CO_2 production for each mole of fermented glucose (McDonald *et al.*, 1991).

Besides decreased DM and increased NDF content, LPPA showed higher EE and ADF concentrations and lower NFC content. These results are assigned with poorly preserved and low nutritive silage. Fermentation losses of the most digestible fraction of silage (Pedroso *et al.*, 2005) result in a higher concentration of low digestible fraction of ensiled material. However, treatment did not affect DM and NDF *in vitro* degradation. Neutral detergent soluble fraction is more digestible than NDF. Although LPPA has increased by 5.5% PDF content relative to other treatments, higher variance naturally observed in in vitro assay resulted in no statistical difference among treatments, even with higher numerical value observed in LB-silage.

Analysing the protein composition of silages, we observed two main issues: (I) LPPA reduced NH_3-N content relative to other treatments, and (II) CHI showed lower protein A-fraction than CON and LB. It is essential to highlight that the CP content of sugarcane is low (28.1 g/kg DM in the present study) and treatment effects on protein composition show limited impact on the nutritional value of silages and animals' performance. Rabelo *et al.* (2019) observed no effect of *L. buchneri* and *L. plantarum* on the NH_3-N concentration of SS. On the other hand, Del Valle *et al.* (2020) observed a linear positive effect of CHI on silage NH_3-N content. This effect was associated with CHI conversion to soluble protonated form when environmental pH is below that of CHI pKa (6.3) (Goy *et al.*, 2009). Therefore, chitosan N was quantified as NH_3-N. However, during protein precipitation analysis, CHI is mainly retained during filtration. Consequently, the A-fraction (soluble) of protein was underestimated. The CHI-treated silos showed a lower A-fraction of protein and higher NH_3-N concentration relative to LPPA-treated ones. In addition, CHI-treated silos showed the lowest BCFA concentration. This result is a consequence of reduced protein degradation once silage BCFA are produced mainly by degradation of branched-chain amino acids (Yang, 2005).

Previous studies have reported positive effects of CHI on chemical composition and *in vitro* degradation (Gandra *et al.*, 2016; Del Valle *et al.*, 2018, 2020). However, CHI did not affect these variables in the present study. These studies reported the use of different CHI specifications and have also reported higher effects on silage fermentation (i.e. reduced ethanol content) and decreased fermentation losses, supporting positive results observed on silage nutritional value.

Conclusion

Although treatments did not affect *in vitro* NDF and DM degradation and silage pH after aerobic exposure, LPPA promotes homolactic fermentation and reduces DM recovery. The LB decreased SS temperature after aerobic exposure, being a more recommended additive to sugarcane ensiling than other additives evaluated in the present study.

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Conflict of interest. The authors declare that are no conflicts of interest to the current manuscript.

Ethical standards. Procedures of the present study were previously approved by the UFSCar Ethics Committee (Approval number 1395120219).

Author contributions. TADV, MC, and JPGM conceived and designed the study. MC, NRP, TMG, and EC conducted data gathering. TADV performed statistical analyses. TADV and JACO wrote the article. TADV, JACO and JPGM critically revised the article.

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