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Author for correspondence:
T. A. Del Valle, E-mail: tiago.valle@ufsm.br

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Chitosan and microbial inoculants in whole-plant soybean silage

J. P. G. de Morais[1], R. Cantoia Júnior[2], T. M. Garcia[1], E. Capucho[1], M. Campana[1], J. R. Gandra[3], Lucas G. Ghizzi[4] and T. A. Del Valle[5]

[1]Department of Biotechnology Vegetal and Animal Production, Agricultural Science Center, Federal University of São Carlos, Araras, 13600-970, Brazil; [2]Umuarama Campus, State University of Maringá, Umuarama, PR 87506-370, Brazil; [3]Institute of Agrarian and Regional Development, Federal University of Southern and Southeastern Pará, Marabá, 68.555-410, Brazil; [4]Department of Animal Nutrition and Production, School of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, 13.635-900, Brazil and [5]Departament of Animal Science, Rural Sciences Center, Federal University of Santa Maria, Santa Maria, RS, 97105-340, Brazil.

Abstract

Whole-plant soybean silage (WPSS) is a potential high-protein roughage source for ruminant diets. However, WPSS can be difficult to ensile and fermentation is a challenge. This study was conducted to evaluate the effect of chitosan and microbial inoculants on fermentation profile, fermentation losses, chemical composition, and in vitro degradation of WPSS. Forty experimental silos (PVC tubing with 28 cm i.d. and 25 cm height) were produced. Soybean plants from 10 plots were ensiled in a completely randomized block design to evaluate the following treatments: (1) control (CON): WPSS without additives; (2) chitosan (CHI): WPSS additive with 6 g/kg DM of chitosan; (3) LBB: WPSS treated with 5.0×10^7 colony-forming units (CFU) of *Lactobacillus buchneri* (NCIM 40788) per kg of fresh matter and (4) LPP: WPSS treated with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of fresh matter. Silos were opened 120 days after ensiling. Microbial inoculants reduced silage pH, whereas LPP-treated silos showed the lowest concentration of NH_3-N, ethanol, butyric, acetic, branched-chain, and propionic organic acids. LBB-treatment decreased lactic acid bacteria (LAB) count relative to other treatments, and LPP-treatment showed the lowest fermentation losses, improving dry matter (DM) recovery. Relative to other treatments, LPP increased silage DM, organic matter, and decreased acid detergent insoluble crude protein (CP), improving DM and neutral detergent fibre *in vitro* degradation. Treatments showed no effect on silage aerobic stability. Thus, LPP-treatment improves fermentation profile, reduces fermentation losses, and increases the nutritional value of WPSS.

Introduction

Although annual legumes are traditionally used for grain production, whole-plant legumes show high dry matter (DM) productively (Mustafa and Seguin, 2003) and could be used to meet ruminant nutritional requirements. Among annual legumes, soybean plants have been highlighted in Brazilian conditions due to the high availability of cultivars, management knowledge, and other technologies. However, soybean harvest is seasonal, necessitating the conservation to use in animal feeding.

Whole-plant soybean has high buffering capacity and low water-soluble carbohydrates content (Ni *et al.*, 2017), impairing its silage fermentation profile. According to Weinberg and Muck (1996), homofermentative lactic acid bacteria improve lactic acid production and inhibit ethanol and ammonia-N (NH_3-N) production, increasing the dry matter recovery by 12%. Especially in tropical conditions, these inoculants may decrease aerobic stability because of insufficient production of short-chain fatty acids that can inhibit yeasts and moulds (Weinberg *et al.*, 1993; Schmidt *et al.*, 2009). More recently, heterofermentative lactic acid bacteria (LAB) inoculants containing *Lactobacillus buchneri* have been used to increase acetic acid production, reduce mould and yeast count, and increase aerobic stability of silages (Weinberg *et al.*, 1999; Filya, 2003).

Silage treatment with chemical additives has been used to modulate fermentation. Chitosan (CHI) is a biopolymer derived from chitin deacetylation and has antimicrobial activity against bacteria and fungi (Kong *et al.*, 2010). Other studies of our research group showed positive effects of CHI addition to sugarcane on silage fermentation, fermentative losses, aerobic stability, and nutritional value of silage (Gandra *et al.*, 2016; Del Valle *et al.*, 2018, 2020). These effects have been associated with the direct inhibition of undesirable microorganisms, such as mould and yeast. Gandra *et al.* (2018) evaluated the association of CHI and a homofermentative LAB inoculant containing *Lactobacillus plantarum* and *Propionibacterium acidipropionici* during the whole plant soybean silage (WPSS) ensiling. Although those authors

reported that CHI had a positive impact on lactate content, DM recovery, and *in vitro* degradation of WPSS, there was no associative effect. Casquete *et al.* (2016) observed that chitosan utilization in food conservation inhibits aerobic and pathogenic bacteria growth. They also reported a synergistic effect with LAB inoculation, which potentially inhibits the growth of deteriorating microorganisms during the aerobic stage of ensiling and improves silage fermentation.

Therefore, we established the hypothesis that CHI or homofermentative LAB inoculant would increase lactic acid count, reduce silage pH and DM losses, while improving the nutritional value and aerobic stability of WPSS in relation to heterofermentative LAB inoculant and control-treated silos. This study was conducted to evaluate the effects of CHI, homofermentative, and heterofermentative microbial inoculant on silage fermentation, count of LAB and mould, fermentation losses, chemical composition, *in vitro* degradation, and aerobic stability of WPSS.

Materials and methods

The trial was performed between March and July 2019, at the Agrarian Sciences Center of São Carlos Federal University (UFSCar), Araras, Brazil.

Soybean, treatments and experimental design

Soybean (cultivars M6410IPRO®, Monsoy – Bayer Crop Science, São Paulo, Brazil) was seeded on 19 November 2018, in ten different plots (almost 1000 m^2 each one). 110 days after the seeding, almost 40 kg of whole-plant soybean were manually harvested from each area at the R6 stage (Coffey *et al.*, 1995) and chopped in a stationary hammer mill (TRF300®, Trapp, Jaguará do Sul, Brazil) to produce four experimental silos (one for each treatment) from each area (plot). The experimental design was a completely randomized block to evaluate the following treatments: (1) CON: WPSS without additives; (2) CHI: WPSS treated with 6 g/kg DM of chitosan; (3) LBB: WPSS treated with 5.0×10^7 colony-formins units (CFU) of *Lactobacillus buchneri* (NCIM 40788, Lasil Cana®, Lallemand Animal Nutrition, Montreal, Canada) per kg of fresh matter; and (4) LPP: WPSS treated with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* (Kera SIL®, Kera Nutrição Animal, Bento Gonçalves, Brazil) per kg of fresh matter. Chitosan had a density of 640 g/L, 883 g/kg DM, 20.0 g/kg of ash, pH of 7.0–9.0, viscosity <200 cPS, and 70 g/kg nitrogen (Polymar Indústria, Fortaleza, Brazil). Chitosan level was based on a previous study of our research group (Del Valle *et al.*, 2020), and inoculant inoculation rates were defined according to the manufacturer's recommendations. Each silo material was individually weighted, manually mixed, and randomly allocated to one silo (PVC tubing with 28 cm i.d, 25 cm height, and equipped with *Bunsen* valve to avoid gas penetration).

Procedures and sampling

Before the treatments were applied, one sample from each area ($n = 10$) was sampled to evaluate chemical composition. The particle size of ensiled WPSS was analysed using the Penn State Particle Separator (Maulfair *et al.*, 2011). Buffering capacity was assessed using Playne and McDonald (1966) method. In addition, 5 kg of dried sand was placed at the bottom of silos to collect effluent losses, and a nylon screen was placed to avoid silage sampling contamination. Before and after filling, silos were weighed using a 5-g sensitivity scale (Mettler Toledo, Barueri, Brazil). Additionally, whole silo weight was recorded at 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 days after the ensiling to estimate gas losses (GL) throughout the ensiling period. Silos were opened 120 days after ensiling: topmost and bottom phase (5 cm) was discarded, and the remaining silage was homogenized for sampling.

A silage sample (200 g) was pressed in a hydraulic press (PHE-45®, Engehidro, Piracicaba, Brazil) to obtain silage fluid. Silage pH was evaluated using a digital potentiometer (LUCA-210®, Lucadema, Sao José do Rio Preto, Brazil) and the remained fluid sample was frozen for NH_3-N, ethanol, and organic acids evaluation. Another sample (500 g) was frozen for chemical analysis and *in vitro* degradation assay. 10-g of fresh silage was diluted in 90 ml of physiological solution (NaCl, 9 g/l). For microbiological enumeration, 1 ml was diluted in 9 ml of physiological solution. Five subsequent dilutions were performed. A pour plating method in a 10-fold serial dilution on MRS Agar® (Kasvi, São José dos Pinhais, Brazil) incubated at 30°C for 48 h for LAB colony counts (Briceño and Martínez, 1995) and on potato dextrose agar (Kasvi) incubated at 26°C for 5 d for yeast and mould colony counts (Rabie *et al.*, 1997). Countable CFU results were transformed for $\log_{10}$ CFU/g, and the average was considered to express the result for each silo. To determine aerobic stability, 3 kg of silage was placed without compaction in a plastic bucket (one for each silo; $n = 40$) and stored in a controlled temperature room (17.0°C ± 1.05; mean ± S.D.) for 7 d. Silage pH was evaluated every 24 h and temperature was measured every eight hours, using spit thermometers (K29-5030®, Kasvi Produtos Laboratoriais, Pinhais, Brazil).

Chemical analysis and in vitro assay

Silage fluid was centrifuged at $500 \times \boldsymbol{g}$ for 15 min. Ammonia-N was evaluated by the Kjeldahl method (method 984.13; AOAC, 2000) without acid digestion. The supernatant of the previously mentioned centrifugation was acidified using formic acid (9:1 ratio, v/v). Organic acids were determined using gas chromatography (GC-2010 Plus chromatograph, Shimadzu, Barueri, Brazil), equipped with an AOC-20i auto-sampler, Stabilwax-DA™ capillary column (30 m, 0.25 mm i.d., 0,25 μm df, Restek©) and a flame ionization detector. Sample (1 μL) was injected with a split ratio of 40:1, using Helium as the carrier gas at a linear velocity of 42 cm/s. The injector and detector temperatures were 250 and 300°C, respectively, and the column's initial temperature was 40°C. The method was calibrated using the WSFA-2 standard (Ref. 47056, Supelco©) and ethanol (Ref. 459828, Sigma-Aldrich©) solutions. The chromatogram was analysed using the GCsolution v. 2.42.00 software (Shimadzu©). The lactic acid concentration was analysed using a spectrophotometric method (Pryce, 1969).

Samples frozen for chemical analysis were thawed at room temperature, dried at 60°C for 72 h, and ground in a knife mill to pass through a 1-mm sieve (SL-31, Solab Científica, Piracicaba, Brazil). It was analysed for DM (method 950.15), ash (method 942.05), ether extract (EE; method 920.39), crude protein (CP; 6,25 × N – method 984.13), acid detergent fibre (ADF), and lignin (method 973.18) as described in AOAC (2000) contents. The neutral detergent fibre (NDF) was analysed without alpha-amylase, and sodium sulphite (Van Soest *et al.*, 1991). Non-fibre carbohydrate (NFC) was calculated as follows: NFC (g/kg DM) = 1000 – (NDF + CP + ash + EE). *In vitro*

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degradation of DM and NDF was determined according to Tilley and Terry (1963) modified by Holden (1999). Samples were processed in a knife mill using a 2-mm sieve and placed in non-woven fabric tissue (5 × 5 cm and 100 g DM/m^2; Casali *et al.*, 2008) bags. The bags (three per sample) were incubated for 48 h at 39°C in an *in vitro* incubator (NL162®, New Lab, Piracicaba, Brazil). Each vial received 1.6 l of McDougall (1948) buffer and 0.4 l of fresh ruminal fluid. It was sampled from two Holstein heifers (400 kg of body weight) maintained in a pasture without concentrate. The inoculum was CO_2 saturated before sample introduction, and 40 bags were incubated in each vial. After removal, samples were washed in running tap water and analysed for NDF content, as previously described.

Protein characterization was performed as described in Cornell Net Carbohydrate and Protein System (CNCPS; Sniffen *et al.*, 1992). Non-protein nitrogen (A-fraction) and soluble protein fraction (B1) were determined after buffer solubilization (Roe *et al.*, 1990). True protein fraction was determined after trichloroacetic acid precipitation (Van Soest *et al.*, 1981). Unavailable protein (C-fraction) was defined as acid detergent insoluble crude protein (ADIP). Slowly degradable protein fraction (B3) was obtained by the difference amount of the neutral detergent insoluble protein (NDIP) and ADIP. Rumen fermentable fraction of protein (B2) was calculated by the difference of buffer insoluble protein and NDIP (Sniffen *et al.*, 1992). All the protein fractions were expressed in g/kg of CP.

Calculations and statistical analysis

Gas losses (*GL*), effluent losses (*EL*), and *DM* recovery (*DMR*) were calculated according to Jobim *et al.* (2007):

$$GL\left(\frac{\text{g}}{\text{kg}}\right) = \frac{[WSWB(\text{g}) - WSWA(\text{g})]}{EM\ (\text{kg})}$$

WSWB and *WSWA* are the whole silo weight before and after the storage, respectively, and *EM* is the ensiled matter (fresh or dried).

$$EL\left(\frac{\text{g}}{\text{kg}}\right) = \frac{(ESWA(\text{g}) - ESWB(\text{g}))}{EM\ (\text{kg})}$$

ESWA and *ESWB* is the empty silo weight after and before the storage, respectively.

$$DMR\left(\frac{\text{g}}{\text{kg}\ DM}\right) = \frac{ODM\ (\text{g})}{EDM\ (\text{kg})}$$

where *ODM* is the *DM* at the opening, and *EDM* is the ensiled dry matter.

Calculations and statistical analysis

Data were analysed using the PROC MIXED of SAS (version 9.4, SAS Inst. Inc., Cary, NC, USA) and the following model:

$$Y_{ij} = \mu + T_i + b_j + e_{ij}$$

with $b_j \approx N(0, \sigma_b^2)$ and $e_{ij} \approx N(0, \sigma_e^2)$, where: Y_{ij} is the observed value of the dependent variable; μ is the overall mean; T_i is the fixed effect of treatment ($i = 1-4$); b_j is the random effect of block (area; $j = 1-10$); e_{ij} is the random residual error; N stands for Gaussian distribution; σ_b^2 and σ_e^2 are the variances associated with the random effects of blocks and residue, respectively.

Gas losses throughout the ensiling period and evaluations of pH and temperature after aerobic exposure were evaluated using the following model:

$$Y_{ijk} = \mu + T_i + b_j + \omega_{ij} + P_k + T \times P_{ik} + e_{ijk}$$

with $b_j \approx N(0, \sigma_b^2)$, $\omega_{ij} \approx N(0, \sigma_\omega^2)$, and $e_{ijk} \approx MVN(0, R)$ where: Y_{ijk} is the observed value; μ, T_i, and b_j were previously defined; ω_{ijk} is the error associated with experimental units (silos); P_k is the fixed effect of the period/time ($k = 1-13$ for gas losses throughout ensiling; 1−21 for temperature; and 1−7 for pH after aerobic exposure); $T \times P_{ik}$ is the interaction between treatment and period effects; e_{ijk} is the experimental error; N stands for Gaussian distribution; σ_b^2 and σ_ω^2 are variances associated with blocks and silos, respectively; *MVN* stands for multivariance normal distribution; R is a variance and covariance matrix due to repeated measures. Matrixes (CS, CSH, AR, ARH, TOEP, TOEPH, FA, UN, ANTE) were evaluated using the Bayesian method. Treatment effects were studied using a protected Fisher's means test (LSD) at 5% of probability.

Results

Fresh whole-plant soybeans used in the present study averaged 250 g/kg DM, 472 g/kg NDF, 57.2 g/kg of ether extract, 618 g/kg of DM *in vitro* degradation, and 784 g/kg of particles higher than 8 mm (Table 1). The addition of LPP during the ensiling reduced ($P \leq 0.05$) NH_3-N, ethanol, butyric, acetic, propionic, and branched-chain fatty acid concentration relative to other treatments evaluated in the present study (Table 2). Both microbial inoculated silos (LBB and LPP) had lower ($P \leq 0.05$) pH values than CON and CHI-treated silages. In addition, LBB decreased ($P \leq 0.05$) ethanol concentration when compared to CHI and CON. Silages from CON and CHI treatments showed a similar ($P > 0.05$) fermentation profile.

Between evaluated treatments, LBB inoculation reduced ($P \leq 0.05$) LAB counts, whereas LPP decreased ($P \leq 0.05$) effluent and gas losses as well as improved ($P \leq 0.05$) DM recovery (Table 3). The lowest GL throughout the ensiling process was in LPP-treated silages (72 g/kg), followed by LBB (109 g/kg), which decreased losses in relation to CON and CHI-silos (116 g/kg; Figure 1). Treatments did not affect ($P = 0.627$) mould and yeast counts. LPP-treated silages showed the highest ($P \leq 0.05$) DM and OM concentration, as well as DM and NDF degradation (Table 4). Chitosan increased ($P \leq 0.05$) CP and NDIP relative to other treatments. There was no treatment effect ($P \geq 0.170$) on WPSS NDF, ADF, NFC, and EE, which averaged 510, 341, 171, and 77.5 g/kg, respectively.

CHI-treatment increased ($P \leq 0.05$) C-fraction of protein in relation to microbial inoculant treatments (Table 5). Additionally, LPP reduced C-fraction relative to the control. However, treatments did not affect ($P \geq 0.069$) other protein fractions of silage evaluated in the present study. There was no treatment and time interaction effect ($P \geq 0.560$) on silage pH and temperature after aerobic exposure (Figs 2 and 3, respectively). Average silage pH was higher ($P \leq 0.05$) for CON (5.57) and CHI-treated (5.58) silos in relation to LBB (5.45) and LPP-treated (5.46) silages. Additionally, treatments did not affect ($P = 0.682$) the temperature of silage after aerobic exposure, although the difference relates to environment temperature decreased ($P < 0.001$) across the time up to 168 h.

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Table 1. Composition and buffer capacity of whole-plant soybeans ($n = 10$) at ensiling (g/kg DM, unless stated)

Item	Mean	S.D.
Chemical composition		
Dry matter, g/kg fresh matter	250	14.4
Organic matter	936	18.7
Neutral detergent fibre	472	26.6
Acid detergent fibre	303	17.7
Non-fibre carbohydrates	233	20.2
Crude protein	178	17.9
Ether extract	57.2	9.78
Neutral detergent insoluble protein	16.8	2.75
Acid detergent insoluble protein	7.10	0.893
In vitro degradation		
Dry matter	618	29.4
Neutral detergent fibre	435	47.5
Particle size		
>19 mm	344	67.9
8–19 mm	440	43.1
4–8 mm	167	28.3
<4 mm	48.7	9.87
Buffering capacity, mEq/kg DM	448	26.7

S.D., standard deviation; DM, dry matter.

Discussion

The present study used ten plots to evaluate the effect of additives on WPSS fermentative profile and losses, chemical composition, *in vitro* digestibility, and aerobic stability. Fresh samples showed a low DM content (250 ± 14.4 g/kg), high concentration of CP (178 ± 17.9 g/kg DM), and buffering capacity (448 ± 26.7 mEq/kg DM), which comprises a challenge for silage preservation (Jatkauskas and Vrotniakiene, 2011). These conditions led to poor silage preservation, as demonstrated by high silage pH, butyric acid, and NH_3-N concentrations (Kung Jr *et al.*, 2018). According to Playne and McDonald (1966), a high buffering capacity of soybean forage results from the high concentration of proteins and organic acids (malic, citric, nicotinic, malonic and glyceric) and their salts present in the plant tissues. For instance, alfalfa also is a leguminosae with a buffering capacity of 488 meq/kg DM, and that of maize ranges from 200 to 250 mE/kg of DM (McDonald *et al.*, 1991). Considering WPSS characteristics similarity with other leguminoseae silages, it is possible to consider that high buffering capacity is the main challenge to improving the fermentation profile and nutritional value of silage.

Both microbial inoculants (LBB and LPP) reduced silage pH, whereas chitosan showed no effect on this variable. LPP inoculation reduced WPSS pH, which is most related to lactic acid and buffering capacity (Kung *et al.*, 2018) and has an essential effect of inhibiting undesirable microorganisms that consume lactic acid (Ni *et al.*, 2017). The LAB produce lactic acid as the main end-product of carbohydrates fermentation (Muck, 2010). However, the concentration of the lactic acid was not affected by additives in the present study. Undesirable microorganisms, such as clostridia, may have been active in transforming protein and sugar into NH_3-N and butyric acid in these high moisture silages (Kung *et al.*, 2018). Therefore, NH_3-N and butyric acid concentrations were reduced in LPP-treated silages suggesting a suppression effect of homofermentative strains on clostridia activity.

LBB-inoculant reduced silage pH in relation to the control and did not affect organic acid concentration. Reduced pH could be associated with non-statistical higher ethanol and lower NH_3-N of LBB-treated silos. *L. buchneri*-treated silos often show 0.1 −0.2 units higher pH than untreated silage (Kleinschmit and Kung, 2006), because of the conversion of lactic acid to acetic acid, 1,2-propanediol (1,2PD), and ethanol (Oude-Elferink *et al.*, 2001). On the other hand, LBB increased the concentration of ethanol, acetic and propionic acids at the expense of LPP-silos. The greater production of acetic and propionic acids, rather than

Table 2. Fermentation profile of whole-plant soybean silage treated with chitosan or microbial inoculants

	Treatments[1]					
Item	CON	CHI	LBB	LPP	S.E.M.[2]	*P*-value
pH	5.78[a]	5.80[a]	5.64[b]	5.62[b]	0.021	<0.001
NH_3-N, g/kg N	97.8[a]	97.5[a]	89.5[a]	70.6[b]	7.91	0.001
Ethanol, g/kg DM	30.6[a]	30.6[a]	23.2[b]	12.7[c]	1.01	<0.001
Organic acids, g/kg DM						
Butyric	46.8[a]	46.4[a]	45.2[a]	40.5[b]	1.21	0.029
Lactic	38.3	35.3	41.7	37.1	1.59	0.394
Acetic	23.2[a]	23.4[a]	22.4[a]	11.5[b]	0.67	<0.001
BCFA[3]	11.8[a]	11.7[a]	10.8[a]	8.15[b]	0.49	0.001
Propionic	9.64[a]	9.93[a]	9.90[a]	5.95[b]	0.453	<0.001

[a–c]Fisher's means test at 5% of probability.
[1]Treatments: CON: WPSS without additive; CHI: WPSS with 6 g/kg DM of chitosan; LBB: WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP: WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.
[2]Standard error of mean.
[3]Branched-chain fatty acids.

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Table 3. Fermentation losses and microbial counts of whole-plant soybean silage treated with chitosan or microbial inoculants

	Treatments[1]					
Item	CON	CHI	LBB	LPP	S.E.M.[2]	*P*-value
Lactic acid bacteria, log_{10}/g	6.98[a]	6.98[a]	6.54[b]	7.09[a]	0.061	0.006
Mould and yeast, log_{10}/g	6.52	6.47	6.18	6.39	0.109	0.627
Fermentation losses						
Effluent, g/kg fresh matter	15.5[a]	18.1[a]	16.3[a]	9.78[b]	1.22	0.001
Gas, g/kg fresh matter	34.3[a]	34.0[a]	32.2[a]	23.7[b]	0.43	<0.001
Total, g/kg fresh matter	49.8[a]	52.1[a]	50.2[a]	33.4[b]	1.42	<0.001
Effluent, g/kg DM	61.5[a]	72.4[a]	65.5[a]	39.3[b]	4.78	0.001
Gas, g/kg DM	137[a]	136[a]	136[a]	95.0[b]	2.5	<0.001
Total, g/kg DM	199[a]	209[a]	201[a]	134[b]	5.8	<0.001
DM recovery, g/kg DM	867[b]	873[b]	882[b]	951[a]	8.7	<0.001

[a,b]Fisher's means test at 5% of probability.
[1]Treatments: CON : WPSS without additive; CHI: WPSS with 6 g/kg DM of chitosan; LBB: WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP: WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.
[2]Standard error of mean.

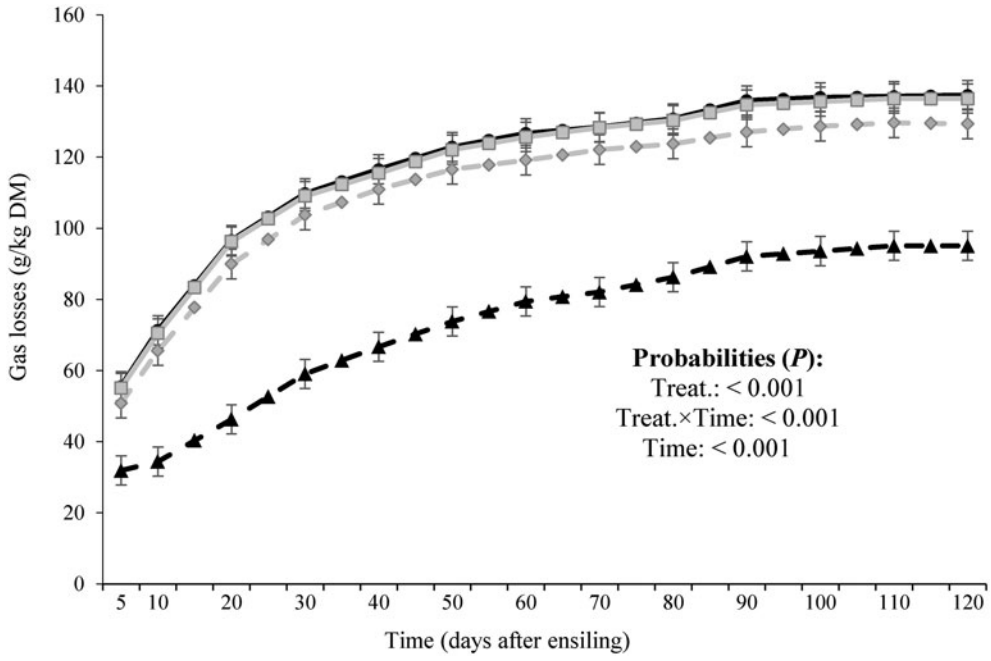

Fig. 1. Gas losses after ensiling of whole-plant soybean silage treated with chitosan and microbial inoculants. Treatments: CON (—●—): WPSS without additive; CHI (—□—): WPSS with 6 g/kg DM of chitosan; LBB (—◇—): WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP(—▲—): WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.

lactic acid, in silages inoculated with *L. buchneri* is well documented in the literature (Pahlow *et al.*, 2003).

According to Senel and McClure (2004), the chitosan bactericidal effect is dependent on pH: the more significant activity is observed at pH values around 4.5. As soybean silage showed a high pH value (range from 5.64 to 5.80), which resulted in no significant effect of CHI. A similar lack of effect on fermentation parameters was observed by Gandra *et al.* (2018). Differently, chitosan positively affected sugarcane silage fermentation and nutritional value, for example, which traditionally showed a low pH environment (Del Valle *et al.*, 2018, 2020). Gandra *et al.* (2018) also observed improved DM recovery and nutritional value on CHI-treated soybean silages. However, it is essential to highlight that most of the CHI effects observed in that study were dependent of microbial inoculant addition; DM content of fresh soybeans was higher than obtained in the present study (342 *vs.* 250 g/kg natural matter) and different chitosan levels were evaluated (5 g/kg natural matter *vs.* 6 g/kg DM). Therefore, Gandra *et al.* (2018) obtained a lower silage pH, which favours the CHI effect on WPSS compared to the present study.

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Table 4. Chemical composition and *in vitro* degradation of whole-plant soybean silage treated with chitosan or microbial inoculants

	Treatments[1]					
Item	CON	CHI	LBB	LPP	S.E.M.[2]	*P*-value
Chemical composition, g/kg DM						
Dry matter, g/kg fresh matter	228[b]	230[b]	231[b]	246[a]	2.4	<0.001
Organic matter	932[b]	932[b]	930[b]	939[a]	1.0	<0.001
Neutral detergent fibre	511	517	516	496	5.1	0.441
Acid detergent fibre	350	337	341	335	3.1	0.342
Non-fibre carbohydrate	172	158	164	188	4.9	0.170
Crude protein	181[b]	197[a]	186[b]	185[b]	1.9	0.024
Ether extract	76.4	74.7	76.6	82.3	2.29	0.267
Neutral detergent insoluble protein	11.2[b]	14.5[a]	11.9[b]	12.1[b]	0.28	0.001
Acid detergent insoluble protein	9.03[ab]	10.0[a]	8.23[bc]	7.69[c]	0.198	0.002
In vitro degradation						
Dry matter	595[b]	599[b]	589[b]	634[a]	5.9	0.007
Neutral detergent fibre	424[b]	415[b]	428[b]	477[a]	6.9	0.004

[a–c]Fisher's means test at 5% of probability.
[1]Treatments: CON : WPSS without additive; CHI: WPSS with 6 g/kg DM of chitosan; LBB: WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP: WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.
[2]Standard error of mean.

Table 5. Protein fractions of whole-plant soybean silage treated with chitosan or microbial inoculants

	Treatments[1]					
Item	CON	CHI	LBB	LPP	S.E.M.[2]	*P*-value
Crude protein	181[b]	197[a]	186[b]	185[b]	1.9	0.024
Protein fractions[3], g/kg						
A	552	585	583	580	8.6	0.490
B1	80.5	55.5	59.8	72.6	4.29	0.163
B2	305	285	293	272	7.1	0.388
B3	12.6	23.4	20.0	24.0	1.55	0.069
C	50.0[ab]	51.2[a]	44.3[bc]	41.7[c]	1.21	0.024

[a–b]Fisher's means test at 5% of probability.
[1]Treatments: CON: WPSS without additive; CHI: WPSS with 6 g/kg DM of chitosan; LBB: WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP: WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.
[2]Standard error of mean.
[3]According to Sniffen *et al.* (1992).

Ni *et al.* (2017) reported a yeast number of 10^6 CFU/g of fresh matter. Although we had no treatment effect on mould and yeast counts, the treatment average ranged from 6.18 to 6.52 $\log_{10}$/g of fresh matter. High ethanol production has been associated with mould and yeast growth (Muck, 2010). According to Kung Jr *et al.* (2018) excessively high acetic acid is observed in high moisture silages that show unwanted fermentation, dominated by enterobacteria, clostridia, or heterolactic acid bacteria (McDonald *et al.*, 1991). The critical pH value for controlling Enterobacter growth in silage at 25% DM is 4.35 (Weissbach and Honig, 1996). Enterobacteria could ferment lactic to acetic acid and other products, causing loss of nutritive value (Ni *et al.*, 2017). Heterofermentative LAB and species of Enterobacterias produce a mix of fermentation products, in which ethanol may represent 50% of them (Mc Donald *et al.*, 1991). Typically, DM gas losses are linked to ethanol production due to carbon dioxide formed during ethanol fermentation (Driehuis and Wikselaar, 2000).

LPP-treated silage showed the highest DM content and a considerably higher DM recovery. Although *L. buchneri* has been extensively used to produce acetic from lactic acid (Kung *et al.*, 2018), it was not observed in the present study. In clostridial silage, epiphytic heterolactic fermentation prevails, which might minimize the effect of these inoculated bacteria. The production of acids during the aerobic stage of ensiling favours the growth of a more acid-tolerant LAB. When the substrate is not limited, LAB growth reduced the silage pH and produced stable silage. If the substrate is limited, the enterobacteria and clostridia may

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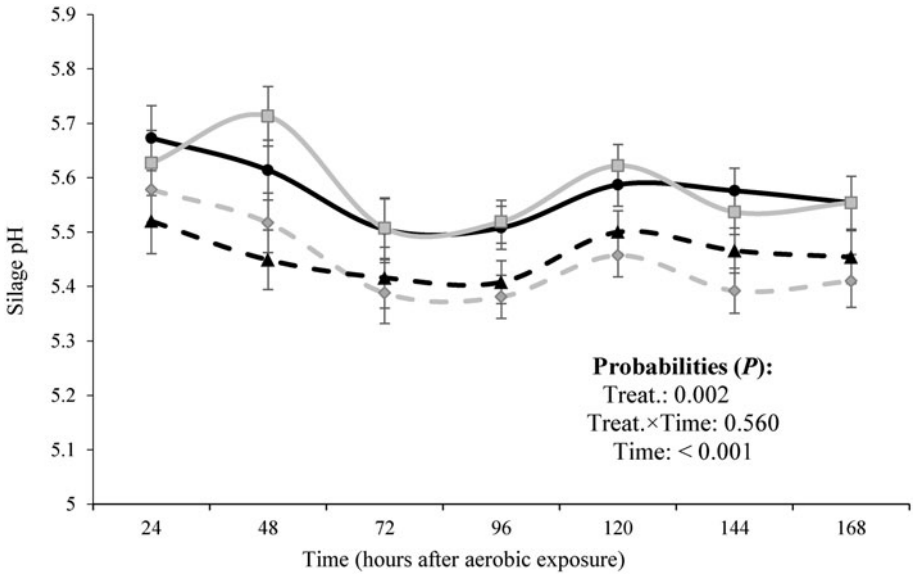

Fig. 2. Silage pH after aerobic exposure of whole-plant soybean silage treated with chitosan and microbial inoculants. Treatments: CON (●): WPSS without additive; CHI (□): WPSS with 6 g/kg DM of chitosan; LBB(◆): WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP(▲): WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.

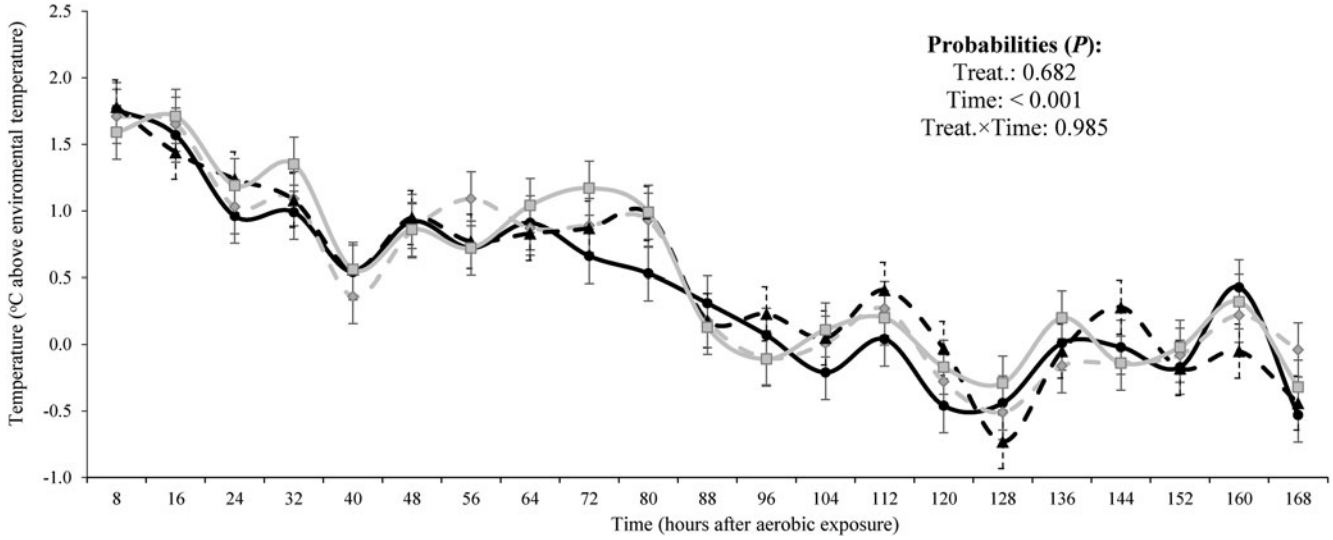

Fig. 3. Silage temperature after aerobic exposure of whole-plant soybean silage treated with chitosan and microbial inoculants. Treatments: CON (●): WPSS without additive; CHI (□): WPSS with 6 g/kg DM of chitosan; LBB (◆): WPSS with 5.0×10^7 CFU/kg fresh matter of *Lactobacillus buchneri*; LPP (▲): WPSS with 1.6×10^8 CFU of *Lactobacillus plantarum* and 1.6×10^8 CFU of *Pediococcus acidilactici* per kg of natural matter.

not be suppressed and may also grow (Rooke and Hatfield, 2003). We evaluated oven-dry matter content in the present study. This method could provide biased results (Daniel *et al.*, 2013) because higher DM content observed in LPP-silages was linked with lower organic acid (except lactic) concentration.

Reduced NH_3-N and ethanol production are associated with decreased EL in LPP-treated silages due to a more desirable fermentation profile than CON-treated silos. According to Muck (2010), yeasts, moulds, and acetic acid bacteria can grow on silage in aerobic conditions, using fermentation products and residual sugars to produce carbon dioxide, water and heat. Water could be drained from the silage, resulting in increased EL. It is important to highlight three consequences of these effluent losses: (1) effluents are produced from the most nutritive fractions (soluble sugars, protein) of ensiled material (Buxton *et al.*, 2003), and reduced effluent production improves the nutritional value of silage, as observed by increased in vitro degradation of LPP-silages in the present study; (2) as effluent production is a physical process that occurred as a consequence of higher moisture content of silage, increased effluent production is linked with depressed silage DM content; (3) effluent has a very high biochemical oxygen demand, being one of the most concentrated farm pollutants (Buxton et al., 2003).

Heterofermentative LAB inoculant had no effect on WPSS chemical composition and in vitro degradation. Filya (2003) has already observed no effect of *L. buchneri* on *in situ* DM, OM and NDF degradability of maize and sorghum silages. On the other hand, CHI increased CP and NDIP. It is essential to highlight that chitosan has 438 g/kg of CP, mostly insoluble in a neutral condition. In acid conditions, a molecular dissociation and solubilization significantly increase (Goy *et al.*, 2009). However, microbial inoculants reduced the C-fraction of protein in relation

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to CON and CHI. We can associate this effect with reduced fermentation losses observed in inoculated-silages, diluting the proportion of low degradable protein fraction.

There was no treatment and time interaction effect on silage pH after aerobic exposure. The differences at the opening were observed throughout the aerobic evaluation. We also observed an unexpected behaviour of silage pH after aerobic exposure. Silage pH started from 5.52 to 5.67 and, after seven days of evaluation, found values between 5.41 and 5.55. According to Parra *et al.* (2019), reduced soybean silage pH after aerobic exposure could be associated with the degradation of alkalizing substances, such as proteins.

Similarly, there was no treatment effect on silage temperature after aerobic exposure. Although it was expected that LBB could improve the aerobic stability of silage, it was not observed. The buffering capacity of Leguminosae is higher than grasses (Wilkinson, 2005). Evaluating 264 legume silages, Pahlow *et al.* (2003) observed that 89% of them were stable 156 h after aerobic exposure. Therefore, as previously discussed, undesirable fermentation has a considerable impact on this variable, resulting in no effects of treatments evaluated in the present study.

Conclusion

Homofermentative LAB inoculant containing *Lactobacillus plantarum* and *Pediococcus acidilactici* reduces fermentation losses and improves WPSS fermentation profile and nutritional value WPSS, but with no effect on aerobic stability. Heterofermentative LAB inoculation and chitosan have no positive effect on WPSS production.

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Conflict of interest. The authors declare that there 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).

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