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Whole-plant soybean ensiling with chitosan and homolactic microbial inoculant: fermentative profile, aerobic stability, and sheep intake and digestibility

Ensilagem de planta inteira de soja com quitosana e inoculante microbiano homolático: perfil fermentativo, estabilidade aeróbia e consumo e digestibilidade em ovinos

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ABSTRACT

This study aimed to evaluate the effects of chitosan and homolactic microbial inoculant on fermentative losses, chemical composition, fermentative profile, and aerobic stability of whole-plant soybean silage (WPSS). Additionally, it was evaluated nutrients intake and digestibility of sheep fed increasing levels of WPSS. Thirty experimental silos were randomly allocated to one of the following treatments: 1) CON: control, WPSS without additives; 2) LPPA: WPSS with *Lactobacillus plantarum* and *Pediococcus acidilactici*;

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and 3) CHI: chitosan, WPSS with 5 g/kg of chitosan. Ten male sheep were used to evaluate increasing dietary levels of WPSS: 0, 200, 400, 600, and 800 g/kg of diet dry matter (DM). Additives increased silage lactic acid bacteria and decreased the count of mold and yeast, gas, and total losses. Silages treated with additives had lower pH, NH_3-N, and ethanol concentrations and higher lactic and propionic acids relative to CON. LPPA-treated silos showed higher organic matter and non-fiber carbohydrates content than CHI-ones. Additives increased the aerobic stability of WPSS. The addition of WPSS in sheep diets linearly increased nutrients intake and digestibility. Chitosan and LPPA improve WPSS fermentation, aerobic stability, and nutritional value. The WPSS in substitution to Cynodon hay increases sheep feed intake and nutrients digestibility.
Keywords: fermentative losses, lactic acid, legume silage, neutral detergent fiber, silage pH.

RESUMO

Este estudo objetivou avaliar os efeitos da adição de quitosana e inoculante homolático sobre as perdas fermentativas, composição química, perfil fermentativo e estabilidade aeróbia da silagem de planta inteira de soja (SPIS). Em adição, foi avaliado o consumo e a digestibilidade de nutrientes em ovinos alimentados com dietas contendo níveis crescentes de SPIS. Trinta silos experimentais foram aleatoriamente alocados a um dos seguintes tratamentos: 1) CON: controle, SPIS sem aditivos; 2) LPPA: SPIS com *Lactobacillus plantarum* e *Pediococcus acidilactici*; e 3) QUI: quitosana, SPIS com 5 g/kg de quitosana. Dez ovinos machos foram usados para avaliar os níveis dietéticos de SPIS: 0, 200, 400, 600 e 800 g/kg da matéria seca (MS). Os aditivos aumentaram a contagem de bactérias láticas e reduziram a contagem de fungos e leveduras e as perdas fermentativas totais da SPIS. Silagens tratadas com aditivos tiveram menores pH, N-NH_3 e etanol e maiores concentrações de ácido lático e propiônico, quando comparadas ao tratamento controle. Silos tratados com LPPA apresentaram maiores teores de matéria orgânica e carboidratos não fibrosos do que aqueles do tratamento QUI. Os aditivos aumentaram a estabilidade aeróbia da SPIS. A adição de SPIS na dieta de ovinos aumentou linearmente o consumo e a digestibilidade dos nutrientes. Quitosana e inoculante microbiano homolático melhoram a fermentação, estabilidade aeróbia e o valor nutricional da SPIS. A substituição de feno de Cynodon por SIPS aumenta o consumo e a digestibilidade dos nutrientes em ovinos.
Palavras-chave: ácido lático, fibra em detergente neutro, perdas fermentativas, pH da silagem, silagem de leguminosa.

Introduction

Whole-plant soybean is rich in protein and vitamin and a promising green fodder source to feed ruminants (JAHANZAD et al., 2014). However, the soybean harvest is seasonal, highlighting the importance of conservation (NI et al., 2017). Ensiling is one of the most traditional conservation practices based on lactic acid fermentation under anaerobic conditions (MCDONALD et al., 1991). After oxygen uptake, lactic acid is produced during ensiling, using water-soluble carbohydrate (WSC) as the primary

substrate (MUCK, 2010). However, whole-plant soybeans have a low level of dry matter (DM) and WSC, resulting in an unpleasant fermentation (NI et al., 2017).

Driehuis et al. (2001) evaluated microbial inoculants in ryegrass silage containing different bacteria. *Lactobacillus plantarum* and *Pediococcus pentosaceus* inoculation improved fermentation conditions. On the other hand, chitosan has been used as an additive in sugarcane silage (GANDRA et al., 2016; DEL VALLE et al., 2018). Gandra et al. (2018) studied chitosan addition in whole-plant soybean silage (WPSS) and observed an increased count of lactic acid bacteria and a positive effect on *in vitro* degradation. To the best of our knowledge, there is no study evaluating chitosan instead of microbial inoculant on WPSS fermentation, chemical composition, and aerobic stability. Therefore, we hypothesized that microbial inoculant or chitosan addition in WPSS reduces silage losses, increases lactic acid and DM degradation of silages.

Protes et al. (2018) evaluated whole-plant soybean silage in replacement to sorghum silage. Soybean silage provides the same animal performance, carcass traits, and economic benefit compared with the sorghum silage diet. Evaluating soybean addition in dairy cows' diets, instead of corn silage as a roughage source, Ghizzi et al. (2020) observed reduced feed intake and animal performance. However, our previous studies evidenced that WPSS has a considerable high in vitro NDF and DM degradation (GANDRA et al., 2018). Our second hypothesis is that increasing WPSS levels, instead of Cynodon hay, in sheep diets could increase feed intake and nutrients digestibility. The present study aimed to evaluate the effects of chitosan and homolactic microbial inoculant effects on fermentative losses, chemical composition, fermentative profile, and aerobic stability of WPSS. Additionally, we assessed the impact of increasing levels of WPSS in sheep diets on nutrients intake and digestibility.

MATERIALS AND METHODS

This trial was conducted between January and April 2018 at the Ruminant Nutrition Laboratory of the Federal University of Grande Dourados, Dourados – MS, Brazil

1 Harvesting, Treatments, and Ensiling

Soybean (cultivar GMX Cancheiro RR; GMX) was cultivated in an experimental farm divided in 30 locations within a 5-ha plot until reaching the R6 stage at 105 d (COFFEY et al., 1995). Approximately 200 kg of soybeans from each location was manually harvested (ground level) and chopped to a theoretical cut of 10 mm using a stationary cutter.

Thirty experimental mini-silos (plastic buckets, 30 cm in height, and 30 cm in diameter) provided with Bunsen valves were randomly distributed. Sand (2,000 grams) was placed in the bottom of the experimental silos and separated from forage with a nylon mesh screen (500 µm) to drain effluents. The additives were applied individually on the whole soybean plant assigned for each bucket to generate correct replications. Forage was added to the buckets at a compaction rate of 650 kg/m^3, and silos were sealed, weighed, and stored at room temperature (26.2 ± 1.3°C; mean ± SD) for 100 d.

Experimental treatments consisted of 1- CON (no additives); 2- LPPA (2 g/t of fresh forage microbial inoculant, Bactosilo® Master Tropical, Lallemand Animal Nutrition, Aparecida de Goiania, Brazil) and 3- CHI (5 g/kg of fresh forage chitosan). Microbial inoculant was composed of *Lactobacillus plantarum* 4.0 × 10^{10} CFU/g + *Pediococcus acidilactici* 10^{10} CFU/g. For LPPA treatment, the inoculant was diluted in water (2 g/L) and sprayed on the forage, and in all silos, water was added in the same proportion as the LPPA. Chitosan presented the following technical specifications: an apparent density of 0.64 g/mL, 2.0% of ash, 7.0 to 9.0 pH, viscosity <200 cPs, and deacetylation level of 95% (Polymar Industria e Cia. Imp. And Exp. LTDA, Fortaleza, Brazil). Chitosan was top-dressed and hand-mixed with fresh forage before forage was added into the silos.

2 Microbiological Quality

At the time of opening the experimental silos, samples of 100 grams were collected from each silo in its intermediate layer. Ten grams from samples were diluted in sterilized sodium chloride solution (0.9%, 90 mL), and a serial dilution was performed. Microorganism counts were carried out in triplicate through decimal dilution series in plates with De Man, Rogosa, Sharpe agar for LAB (BRICEÑO & MARTINEZ, 1995), nutrient agar for aerobic and anaerobic bacteria (48 h of incubation at 30°C), and potato dextrose agar (120 h of incubation at 26°C) for mold and yeast as described by Rabie et al. (1997). The absolute values were obtained as colony-forming units and then log-transformed.

3 Fermentative losses

Experimental silos were weighed to determine gas losses. Effluent losses were calculated based on the difference between the weight of silo assembly (plastic bucket, nylon screen, and sand layer) before the storage and weight of silo assembly after 100 d.

Gas losses (GL), effluent losses (EL) and dry matter recovery (DMR) were calculated according to Jobim et al. (2007), as follows:

$$GL\left(\frac{g}{kg}DM\right) = \frac{SWE(g) - SWO(g)}{DME(kg)}$$

in which: SWE is the silo weight at the ensiling, SWO is silo weight at the opening, and DME is total DM ensiled.

$$EP\left(\frac{g}{kg}DM\right) = \frac{WSAO(g) - WSAE(g)}{DME(kg)}$$

where: WSAO is the weight of silo assembly after the opening (g), and WSAE is the weight of silo before the ensiling (g).

$$DMR\left(\frac{g}{kg}\right) = \frac{DMO\ (g)}{DME\ (kg)}$$

In which: DMO is total DM after the opening of the silo (kg), and DME is total DM before the ensiling (kg).

4 Fermentative Profile

Silage juice was extracted from forage samples using a hydraulic press, and pH was measured using a digital potentiometer (LUCA-210®, Lucadema, Sao José do Rio Preto, Brazil). Silage juice aliquots (2 mL) were mixed with 1 mL of sulfuric acid (1 *N*) for the determination of ammonia nitrogen concentration through the colorimetric method described by Foldager (1977). Organic fatty acids and ethanol were determined as reported by Del Valle et

al. (2018). Briefly, aliquots (1 mL) of silage juice were mixed with formic acid (0.2 mL) in amber glass bottles and frozen until analysis. Volatile fatty acids and ethanol concentrations were determined in a gas chromatograph (Focus GC, Thermo Fisher Scientific Inc., Waltham, MA) equipped with an automatic sample injector (model AS-3000, Thermo Fisher Scientific Inc.), a glass column (2.0 m × 0.5 cm 80/120 Carbopack B-DA/4% Carbowax 20M phase; Sigma-Aldrich, St. Louis, MO), and a flame ionization detector set at 270°C. The chromatograph oven and injector temperatures were set to 190°C and 220°C, respectively. Hydrogen was used as the carrier gas flowing at 30 mL/min. The lactic acid concentration was measured by HPLC (LC-10ADVP Shimadzu HPLC system, Shimadzu Inc., Kyoto, Japan), according to Ding et al. (1995).

5 Chemical Composition and In Vitro Degradation

Samples (1,000 g) of chopped soybean plant were assessed for contents of DM (method 950.15), ash (method 942.05), OM (DM − ash), CP (N × 6.25; method 984.13), and ether extract (EE; method 920.39) according to AOAC International (2000; Table 1). Non-fiber carbohydrate was calculated as NFC = 1,000 − (NDF + CP + EE + ash), all values expressed as grams per kilogram of DM. Neutral detergent fiber (without sodium sulfite), ADF, and lignin (sulfuric acid method) were determined according to Van Soest et al. (1991). The net energy of lactation was estimated, according to NRC (2001). Silage buffering capacity was analyzed according to Playne and McDonald (1966), and the digestion method described by Miller (1998) was used to determine macro-minerals.

Table 1. Chemical composition of whole soybean plant before ensiling

Chemical composition, g/kg DM	
Dry matter, g/kg as fed	394
Organic matter	911
Neutral detergent fiber	512
Acid detergent fiber	381
Crude Protein	210
Non-fiber carbohydrate†	191
Ash	88.7
Lignin	63.1
Ether extract	25.3
Potassium	17.0
Calcium	10.0
Magnesium	5.10
Sulfur	2.20
Phosphor	1.90
NE (MJ/kg DM)†	5.74
Buffering capacity, mEq/kg of DM	527

†Estimated according to NRC (2001).

Dry matter and NDF *in vitro* digestibility were determined using filter bags and artificial rumen incubator (TE-150, Tecnal, Piracicaba, Brazil) according to Tilley and Terry (1963) and adapted by Holden (1999). Briefly, filter bags with samples were incubated for 48 h at 39°C in a buffer-inoculum solution (1,600 mL of buffer solution and 400 mL of rumen inoculum). The rumen inoculum was obtained from two Jersey heifers, fed with corn silage *ad libitum*, and 2 kg of concentrate per day. Samples were performed before the morning fed, using a PCV probe. Jars containing the buffer-inoculum solution were purged with CO_2, and lids had gas relief valves. After the incubation period, the buffer-inoculum was drained from the jars, and the filter bags were gently squeezed against the sides of the jar to remove the gas trapped in the inflated bags. Afterward, bags were rinsed in jars with three changes of warm tap water.

6 Silage Aerobic Stability

During the 6-d period of aerobic stability evaluation, silos were maintained at room temperature (23.3 ± 2.34, mean ± SD), and the temperature of WPSS was measured every eight hours after oxygen exposure using an infrared thermometer (MS6530, Wiltronics Research Pty. Ltd., Victoria, Australia). Besides, samples (200 g) from silos of each treatment were collected every 24 h to assess pH after silo oxygen exposure (Kung et al., 1984). The aerobic stability was defined as the period (h) in which WPSS temperature remained less than 1°C above the room temperature (DRIEHUIS et al., 2001).

7 In vivo nutrients intake and digestibility

Ten castrated lambs (28.7 ± 3.66 kg body weight and 6.4 ± 0.3 mo) were assigned to a 5 × 5 Latin square design trial, consisting of 19-d periods, with the last 5 d for data record and sampling. Diet was formulated for 200 g average daily gain, using Small Ruminants Nutritional System (SRNS) (Table 2). Lambs within each square were randomly assigned to diets containing increasing levels of whole plant soybean silage in the total diet (0, 200, 400, 600, and 800 g / kg of DM). Silage was produced in 200 L tubs (3 tubs for each treatment). Silages were produced as previously described: microbial inoculant (*Lactobacillus plantarum* 4.0 × 10^{10} CFU/g + *Pediococcus acidilactici* 10^{10} CFU/g) was individually weighed (2 g/ton.), diluted in water, and manually mixed with whole-plant soybean silage. Animals were housed in metabolic cages and fed twice daily, at 07:00 and 13:00 h, targeting refusals between 10% to 15%. Samples of feeds and refusals were collected daily during the sampling period and pooled in a composite sample for subsequent chemical analyses.

Table 2. Ingredients and chemical composition of experimental diet

Item	Experimental diets†				
	0	200	400	600	800
Ingredients					
Soybean silage	0.00	200	400	600	800
Cynodon hay	800	600	400	200	0.00

Ground corn	31.5	59.1	104	118	160
Ground whole soybean	121	96.1	50.8	41.8	0.00
Urea	7.90	5.00	4.90	0.00	0.00
Mineral premix‡	39.8	39.8	39.8	39.8	39.8
Chemical composition, g/kg DM					
Dry matter, g/kg as fed	836	741	647	552	457
Organic matter	927	921	917	912	910
Neutral detergent fiber	617	548	476	410	338
Acid detergent fiber	312	303	293	286	276
Crude Protein	155	155	156	155	157
Non-fiber carbohydrate§	14.5	21.4	29.0	35.6	42.9
Ether extract	46.0	43.0	35.0	35.0	29.0
NE (MJ/kg DM)§	2.89	3.22	3.48	3.85	4.10

†Increasing dietary levels of WPSS: 0, 200, 400, 600, and 800 g/kg DM.
‡Contained per kg of product: 120 g Ca, 88.0 g P, 75.0 mg I, 1,300 mg Na, 15.0 mg Se, 12.0 mg S, 3,630 mg Zn, 55.5 mg Co, 1,530 mg Cu, and 1,800 mg Fe.
§Estimated according to NRC (2001).

On days 16–18 of each experimental period, total fecal collections were performed through a metabolic cage device that separates urine from the feces. The feces were weighed every 24 hours of collection, and a 10% aliquot of each collection day was collected for further analysis of the digestibility of DM, CP, NDF, and EE. Samples of silages, dietary ingredients, refusals, and feces were analyzed for DM (method 950.15), crude protein (CP, N × 6.25; Kjeldahl method 984.13), ether extract (EE; method 920.39) according to AOAC (2000) and neutral detergent fiber (without sodium sulfite), according to Van Soest et al. (1991). Nutrient digestibility (NuD) was estimated as:

$$NuD\left(\frac{g}{kg}\right) = \frac{Nu_{intake}(g) - Nu_{Fecal}(g)}{Nu_{intake}(kg)}$$

where Nu_{intake} is the nutrient intake and Nu_{Fecal} is the fecal nutrient excretion.

8 Statistical Analysis

Statistical analyses of silage evaluations were performed using PROC MIXED of SAS (SAS Institute Inc, 2011). Data from the silo experiment were analyzed using the following model:

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

with $e_{ij} \approx N(0, \sigma_e^2)$, where: Y_{ij} is the observed value; μ is the overall mean; T_i is the fixed effect of treatment (i = 1, 2, and 3); e_{ij} is the random residual error (j = 1 to 10); N stands for Gaussian deviation; and σ_e^2 is the variance of error. The treatment effect was analyzed as orthogonal contrasts: (1) (LPPA + CHI) *vs.* CON, and (2) LPPA *vs.* CHI.

Data of nutrients intake and digestibility were analyzed according to the following model:

$$Y_{ijkl} = \mu + S_i + a_{j:i} + T_k + P_l + e_{ijkl},$$

with $a_{j:i} \approx N(0, \sigma_a^2)$; $e_{ijkl} \approx N(0, \sigma_e^2)$, where: Y_{ijkl} is the value of the dependent variable; μ is the overall mean; S_i is the fixed effect of Latin Square (i = 1 and 2); $a_{j:i}$ is the random effect of j^{th} animal within the i^{th} Latin Square (j = 1 to 10); T_k is the fixed effect of treatment (k = 1,

2, 3, 4 and 5); P_l is the fixed effect of the experimental period (l = 1, 2, 3, 4 and 5); e_{ijkl} is the random experimental error; N stands for Gaussian deviation; σ_a^2 is the variance of animals; and σ_e^2 is the variance of error. The treatment effect was analyzed as a polynomial regression. The significance level of 5% was considered for all statistical analyses.

9 Results

The additives increased ($P \leq 0.033$) lactic acid and aerobic bacteria and decreased ($P = 0.001$) the count of mold and yeast (Table 3). Also, silos containing additives had lower ($P \leq 0.044$) gas and total losses, with higher ($P \leq 0.033$) effluent losses and DM recovery, relative to CON silos. Between evaluated additives, CHI increased ($P \leq 0.012$) counts of aerobic bacteria and mold and yeast, and did not affect ($P \geq 0.652$) anaerobic and lactic acid bacteria count, compared to LPPA. Chitosan showed lower ($P \leq 0.042$) gas and total losses, and higher ($P \leq 0.042$) DM recovery, than LPPA. There was no difference ($P \geq 0.342$) among CHI and LPPA on effluent losses.

Table 3. Microbiology and fermentative losses of whole plant soybean silage treated with chitosan and homolactic microbial inoculant

Item	Tratamentos†			SEM	P‡	
	COM	LPPA	CHI		C1	C2
Microbiology, $\log_{10}$ CFU/g						
Aerobic bacteria	5.95	6.69	8.32	0.153	0.001	0.003
Anaerobic bacteria	6.04	5.56	4.60	0.164	0.512	0.652
Lactic bacteria	6.61	8.36	7.89	0.101	0.033	0.653
Mold and yeast	6.89	4.54	5.00	0.112	0.001	0.012
Fermentative losses						
Gas, g/kg fresh matter	22.2	13.7	10.9	0.203	0.044	0.001
Gas, g/kg DM	81.2	49.8	30.2	0.673	0.021	0.067
Effluent, g/kg fresh matter	3.69	4.21	4.09	0.311	0.033	0.342
Effluent, g/kg DM	3.40	4.00	3.80	0.174	0.010	0.563
Total, g/kg DM	84.7	53.8	34.0	0.32	0.001	0.042
DM recovery, g/kg DM	915	946	966	0.32	0.001	0.042

†Treatments: CON (Control), WPSS without additives; LPPA (*Lactobacillus plantarum* 4.0 × 10^{10} CFU/g + *Pediococcus acidilactici* 10^{10} CFU/g); CHI: chitosan, 5 g/kg as-fed.
‡Probabilities: C1: additives effect (CON vs LPPA+CHI); C2: comparison of additives (LPPA vs CHI).

Silages treated with additives had lower ($P \leq 0.021$) pH value, NH_3-N, and ethanol concentrations (Table 4). On the other hand, CHI and LPPA increased ($P \leq 0.012$) lactic, BCFA, and propionic acids, in relation to CON. Additionally, CHI-treated silos had higher ($P \leq 0.038$) NH_3-N, ethanol, and acetic acid, compared to those silos of LPPA treatment. Treatments did not affect ($P \geq 0.234$) silage butyric acid concentration. Additives supply in WPSS reduced ($P \leq 0.011$) silage content of DM and EE, whereas increased the content of CP and

Ca (Table 5). Concerning CHI, the supply of LPPA reduced (P = 0.021) Ca and increased ($P \leq 0.032$) silage content of OM and NFC. However, treatments showed no effects ($P \geq 0.606$) on silage fiber content (NDF and ADF), as soon as the net energy content of WPSS. Although additives increased (P = 0.022) NDF *in vitro* degradation, treatments showed no effects ($P \geq 0.147$) on DM *in vitro* degradation.

Table 4. Fermentative profile of whole plant soybean silage treated with chitosan and homolactic microbial inoculant

Item	Tratamentos†			SEM	P‡	
	CON	LPPA	CHI		C1	C2
pH	3.55	3.46	3.45	0.011	0.003	0.687
NH_3-N, g/kg N	82.6	75.3	80.7	0.65	0.002	0.001
Organic acids, g/kg DM						
Lactic	5.54	6.01	6.78	0.033	0.012	0.232
Ethanol	0.723	0.493	0.566	0.082	0.021	0.038
Acetic	1.40	1.28	1.82	0.015	0.372	0.004
Propyonic	0.070	0.090	0.093	0.021	0.001	0.576
Butyric	0.197	0.123	0.136	0.026	0.659	0.234
BCFA§	0.216	0.278	0.296	0.088	0.001	0.354

†Treatments: CON (Control), WPSS without additives; LPPA (*Lactobacillus plantarum* 4.0 × 10^{10} CFU/g + *Pediococcus acidilactici* 10^{10} CFU/g); CHI: chitosan, 5 g/kg as-fed.
‡Probabilities: C1: additives effect (CON vs LPPA+CHI); C2: comparison of additives (LPPA *vs* CHI).
§Branched-chain fatty acids.

Table 5. Chemical composition of whole plant soybean silage treated with chitosan and homolactic microbial inoculant

Item	Tratamentos†			SEM	P‡	
	CON	LPPA	CHI		C1	C2
Chemical composition, g/kg DM						
Dry matter	353	338	332	7.1	<0.001	0.784
Organic matter	927	931	926	1.0	0.650	0.004
Neutral detergent fiber	401	406	404	2.7	0.887	0.885
Acid detergent fiber	341	339	341	3.4	0.776	0.665
Crude protein	179	198	194	2.6	0.011	0.342
Non-fiber carbohydrate	352	350	341	7.4	0.543	0.032
Lignin	63.5	63.6	65.6	1.26	0.432	0.776
Ether extract	31.8	25.1	20.9	3.02	0.001	0.332
Potassium	13.2	12.9	13.2	0.11	0.321	0.342
Calcium	8.00	8.30	8.90	0.11	0.017	0.021
Magnesium	4.40	4.40	4.34	0.11	0.654	0.340
Sulfur	1.96	1.63	1.86	0.11	0.543	0.421
Phosphor	2.28	2.48	2.47	0.12	0.876	0.760
NE, MJ/kg DM§	6.03	6.07	6.03	0.046	0.998	0.606

In vitro degradation, g/kg						
Dry matter	641.2	648.5	647.6	4.45	0.147	0.544
Neutral detergent fiber	542.5	555.8	557.6	3.41	0.022	0.484

†Treatments: CON (Control), WPSS without additives; LPPA (*Lactobacillus plantarum* 4.0 × 10^{10} CFU/g + *Pediococcus acidilactici* 10^{10} CFU/g); CHI: chitosan, 5 g/kg as-fed.
‡Probabilities: C1: additives effect (CON after aerobic exposure, compared to LPPA+CHI); C2: comparison of additives (LPPA CHI. The addition of WPSS in sheep CHI).
§Estimated according to NRC(2001).

Additives increased ($P \leq 0.023$) the time of aerobic stability and average pH after aerobic exposure (Table 6). Comparing additives, LPPA increased ($P = 0.001$) aerobic stability period and reduced ($P \leq 0.026$) average pH and DM of silage diets linearly increased ($P \leq 0.044$) DM, OM, and CP intake and digestibility (Table 7). Although WPSS quadratically affected ($P = 0.033$) NDF intake, there was ($P = 0.021$) linear positive effect on NDF digestibility. Maximal NDF intake was observed using 389 g/kg DM of WPSS.

Table 6. Aerobic stability of whole plant soybean silage treated with chitosan and homolactic microbial inoculant

Item	Tratament†			SEM	*P*‡	
	CON	LPPA	CHI		C1	C2
Temperature, °C						
Maximun	24.9	25.2	26.4	0.12	0.265	0.432
Sum (5 days)	325	322	346	1.09	0.123	0.262
Average	19.6	21.6	24.2	0.33	0.012	0.022
Time, h						
Stability	46.2	110	77.8	1.07	0.002	0.001
pH	3.86	3.67	4.21	0.442	0.023	0.001
Dry matter	376	352	361	0.36	0.342	0.026

†Treatments: CON (Control), WPSS without additives; LPPA (*Lactobacillus plantarum* 4.0 × 10^{10} CFU/g + *Pediococcus acidilactici* 10^{10} CFU/g); CHI: chitosan, 5 g/kg as-fed.
‡Probabilities: C1: additives effect (CON vs LPPA+CHI); C2: comparison of additives (LPPA *vs* CHI).

Table 7. Nutrients intake and digestibility of finishing sheep fed with increasing levels of WPSS, replacing cynodon hay

Item	Experimental diets†					SEM	*P*‡	
	0	200	400	600	800		Linear	Quadratic
Intake, g/d								
Dry matter	714	1052	1172	1285	1342	72	0.002	0.284
Organic matter	662	967	1073	1168	1220	65	0.003	0.348
Crude protein	111	163	183	198	210	11	0.014	0.541
Neutral detergent fiber	441	575	557	525	453	26	0.321	0.033§
Apparent digestibility, g/kg								

Dry matter	526	550	557	573	608	1.18	0.017	0.719
Organic matter	529	553	562	588	615	2.67	0.026	0.812
Crude protein	658	676	707	726	757	1.78	0.044	0.519
Neutral detergent fiber	502	524	555	579	591	2.09	0.021	0.426

†Incresing levels of dietary WPSS (g/kg DM).
‡Probabilities: linear and quadratic effect of WPSS level.
§NDF intake (g/d) = 454 + 0.59 WPSS – 7.60 × 10^{-4} $WPSS^2$. Maximum point: 389.0 g/kg DM.

DISCUSSION

The antifungal effect of chitosan and derivates have been well documented (CHAPARRO-HERNÁNDEZ et al., 2015; SAEED et al. 2019). Mold and yeasts are undesirable microorganisms of silages that provide silage deterioration, and its inhibition could improve substrate disponibility to bacteria development. Therefore, chitosan has been associated with a positive effect on counts of lactic acid and aerobic bacteria (GANDRA et al., 2016; 2018). On the other hand, anaerobiosis and acidification are considered key points to mold and yeast inhibition (PAHLOW et al., 2003). Homolactic bacteria inoculant anticipates lactic acid bacteria establishment and provides acidification, with other beneficial effects on silage fermentation, especially in low-water soluble carbohydrates materials (OLIVEIRA et al., 2017). Therefore, both additives similarly increased lactic acid in the present study. Additionally, LPPA even showed a more evident negative effect on mold and yeast count, besides both treatments reduced counts of mold and yeast.

Lactic acid bacteria have been commonly used to improve lactic acid fermentation, inhibit harmful epiphytic microbes, and preserve the nutritional value of ensiled material (ARRIOLA et al., 2015; SILVA et al., 2016). According to Muck (2010), lactic acid is the goal end product of silage fermentation, due to more substantial acidification power of lactic (pKa 3.86) than acetic acid (pKa 4.76). Therefore, additives reduced silage pH and fermentative losses of WPSS, in the present study. Oliveira et al. (2017) reported that LAB inoculation of forages with low WSC, such as alfalfa, tropical and temperate grass silages, reduces pH and improves DM recovery of silages. According to Driehuis and van Wikselaar (2000), a fast decline of silage pH reduces the risk of undesirable fermentations by enterobacteria or clostridia, which are mainly aerobic bacteria, and can increase protein degradation. Branched-chain fatty acids (valeric, isovaleric, and isobutyric) are produced from proteolysis and metabolism of branched-chain fatty acids (valine, leucine, and isoleucine; CROWN, MARZE, & ANTONIEWICZ, 2015). As previously discussed, LPPA probably reduced silage pH faster than CHI. Therefore, CHI increased NH_3-N and reduced BCFA, relative to LPPA, due to a higher count of aerobic bacteria.

According to Kung Jr. et al. (2018), some species of clostridia can ferment both carbohydrates and proteins, which are converted into ammonia and biogenic amines. In the present study, additives reduced NH_3-N and significantly improved the CP content of the silage.

The EE uptake during ensiling is near zero, and silage EE concentration improves when fermentation losses increase. Therefore, reduced fermentative losses observed on additive-treated silos resulted in a higher EE content relative to silos of CON treatment. The high moisture content (high than 700 g/kg) and elevated pH of silages favor clostridial fermentation (KUNG Jr. et al., 2018). However, additives decreased DM content of silages. It is known that a higher LAB fermentation rate of silages increases water activity (GREENHILL, 1964), and LAB inoculation is more effective in high moisture silages (DRIEHUIS et al., 1997). The authors agree that this result is linked with increased effluent losses observed on additives-treated silages in the present study. It is interesting to highlight that either effect (on EE and DM) was insufficient to affect additives positive effect on silage DM recovery.

In general, additives showed a more significant negative effect on ethanol concentration and reduced fermentative losses, improving DM recovery. Additionally, previously discussed inhibition of secondary fermentation shows an important effect on fermentative losses and DM recovery (BORREANI et al., 2018). Chitosan-treated silos showed higher ethanol concentration, which is prone to an increased count of mold and yeast, in relation to LPPA. However, CHI had lower gas fermentative losses than LPPA. Although alcoholic fermentation could be a relevant source of fermentative losses in WSC-rich crops, like sugarcane (PEDROSO et al., 2005), ethanol concentration observed in the present study is remarkably lower than observed in those studies. According to Borreani et al. (2018), microorganisms other than LAB play a significant role in fermentation DM loss by carbon dioxide synthesis. This is particularly true for yeasts producing ethanol from glucose (e.g., sugarcane silage) or clostridia producing butyrate from lactate or glucose. In legume silages, in which ethanol production is lower important, other secondary fermentation end-products gain relevance.

Weinberg et al. (1993) studied the LAB inoculation effect on the aerobic stability of wheat, hedysarum, corn, and sorghum silage. These authors associated aerobic deterioration of inoculated silages with high levels of residual WSC and lactic acid, and lack of other organic fatty acids. On the other hand, several studies (GANDRA et al., 2016; DEL VALLE et al., 2018; GANDRA et al., 2018) have been reported a positive effect of chitosan on aerobic stability of silage, by a direct effect on yeast growth. In the present study, additives increased the aerobic stability of silage. However, LPPA showed higher aerobic stability than CHI. Higher short-chain fatty acids content of CHI-treated silos seems to inhibit deterioration and favor stability after aerobic exposure.

When WPSS was added in sheep diets, instead of *Cynodon* hay, it was observed a linear increase in nutrients digestibility. It highlights the higher nutritional value of WPSS, related to Cynodon hay. Intrinsic characteristics of diet have been considered the restrictive factor for rumen degradation, as a large and non-limiting enzymatic pool would exist in the rumen (DETMANN et al., 2008). Considering that digestion and passage are concurrent events (NOCEK, 1988), diets with lower NDF digestibility restrict animal feed intake. In the present

study, besides higher nutrients digestibility, diets with increasing levels of WPSS had lower NDF content. Therefore, it was observed linear positive effects on feed intake. The quadratic effect observed on NDF intake could be considered a consequence of great DM intake depression of CON-diet, and low NDF content of 800-diet.

CONCLUSION

Chitosan and LPPA addition in WPSS reduces fermentative losses and silage pH, improving crude protein content and aerobic stability of silage. Chitosan reduces fermentative gas losses and shows lower aerobic stability, compared to LPPA. Besides, increasing levels of WPSS in sheep diets linearly increases feed intake and nutrients digestibility.

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