Soybean whole-plant ensiled with chitosan and lactic acid bacteria: Microorganism counts, fermentative profile, and total losses

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ABSTRACT

Chitosan is a biopolymer derived from chitin deacetylation, present in the exoskeleton of crustaceans and insects. Chitosan has been evaluated as rumen modulator and silage additive due to its antimicrobial properties. The objective of this study was to determine the effects of both chitosan and a bacterial additive on microbiological quality, chemical composition, nutrient in vitro degradation, fermentative profile, and total losses of whole-soybean plant silage (SS) harvested at R6 stage. Four treatments in a factorial arrangement were randomly assigned to 40 experimental minisilos as no additives (CON), 8 g/t fresh forage of microbial inoculant (INO; Kera SIL, Kera Nutrição Animal, Bento Gonçalves, Brazil); 5 g/kg of fresh forage chitosan (CHI); and CHI + INO. Microbial inoculant was composed of *Lactobacillus plantarum* (4.0×10^{10} cfu/g) and *Propionibacterium acidipropionici* (2.6×10^{10} cfu/g). The CHI and INO alone increased counts of lactic bacteria and anaerobic bacteria and decreased counts of mold and yeast in SS. The CHI or INO alone increased in vitro degradation of dry matter, crude protein, and neutral detergent fiber, and decreased nonfiber carbohydrate content of SS. Chitosan increased NH_3-N and lactate concentrations and decreased ethanol concentration in SS. The CHI increased dry matter recovery from SS; INO increased silage aerobic stability. The combination of CHI+INO showed the lowest value of gas losses. In general, the combination of CHI and INO had small positive effects on gas losses of SS; however, both CHI or INO alone improved nutrient in vitro degradation and decreased mold and yeast in SS. Chitosan or INO utilization improves SS quality.
Key words: chitin, *Lactobacillus plantarum*, legume silage, *Propionibacterium acidipropionici*

INTRODUCTION

Soybeans were initially used as a hay or forage crop (Smith and Huyser, 1987), but production has shifted over time toward grain because of an increase in cereal prices. Previously, farmers ensiled soybeans to avoid forage shortage when adverse weather (i.e., early frost) jeopardizes the potential of high-quality grain production (Undersander et al., 2007). Soybeans have also been ensiled mixed with tropical grasses to improve silage composition in terms of protein content (Stella et al., 2016). Currently, cultivars are bred to improve forage or silage production of soybeans (Devine and Hatley, 1998; Sheaffer et al., 2001); these cultivars can replace alfalfa silage without affecting feed efficiency and ECM of cows producing 35 kg/d of milk (Vargas-Bello-Pérez et al., 2008).

The soybean plant has low DM content and high contents of protein, oil, and ash that are positively associated with effluent production and buffering capacity. Nevertheless, studies have shown soybeans can be well preserved as silage (Mustafa and Seguin, 2003). Unlike corn, sorghum, or alfalfa silages, little is known in terms of enhancing fermentation profile of whole-plant soybean silage (**SS**) through additives. Nkosi et al. (2016a) reported an improvement in the aerobic stability and lactic acid content after adding a mixture of lactic acid bacteria (**LAB**) and fibrolytic enzymes to SS. Recently, researchers demonstrated that SS ensiled with molasses and homofermentative LAB (*Lactobacillus plantarum* and *Pediococcus pentosaceus* at application ratio of 4:1 and 10^6 cfu/g) exhibited higher production of lactic acid and acetic acid, as well as lower pH and butyric acid production compared with control group (Ni et al., 2017). The positive effects of LAB on fermentative profile of different silages (corn, sorghum, and alfalfa) are described elsewhere (Filya, 2003; Filya et al., 2007; Contreras-Govea et al., 2013). However, the application of homofermentative LAB has been positively associated with aerobic spoilage of silages (Weinberg et al., 1993), either due to low production of organic

acids, which is not enough to inhibit aerobic yeasts and molds (Moon, 1983), or due to utilization of lactic acid by lactate-assimilating yeasts (Wohlt, 1989). To minimize the aerobic spoilage of silages, LAB have been inoculated along with propionic acid bacteria such as *Propionibacterium acidipropionici* (Filya et al., 2004). Propionic acid bacteria ferment sugars and lactate into acetate and propionate, which can inhibit the growth of yeasts and molds (Moon, 1983).

Chitosan is a biopolymer derived from the deacetylation of chitin, the second most abundant natural polymer that composes the exoskeleton of crustaceans and insects (Senel and McClure, 2004). Chitosan has demonstrated antimicrobial activity against bacteria and fungi, being able to completely inhibit dimorphic fungi proliferation in in vitro studies (Senel and Mc-Clure, 2004; Olicón-Hernández et al., 2015). For instance, sugarcane silage-treated chitosan showed longer aerobic stability and higher concentration of lactic acid in comparison with control or compared with sugarcane silage treated a mixture of *Lactobacillus buchneri* and *Bacillus subtilis* (Gandra et al., 2016a).

Despite the positive effects of LAB inoculants or chitosan on forage preservation, few studies have evaluated their combination; thus, it is unknown whether chitosan has any detrimental effects on LAB establishment and consequently on SS fermentation. Recently, Del Valle et al. (2018) reported a positive interaction effect between chitosan and inoculant on fermentative losses and DM recovery (**DMR**) of sugarcane silage. The evidence that chitosan decreases yeasts and molds and increases lactic acid concentration in silage (Gandra et al., 2016a) raises the hypothesis that chitosan could increase the substrate availability (lactic acid) for *P. acidipropionici* fermentation and potentiate its effects on aerobic stability. Therefore, the objective of our study was to evaluate the effects of chitosan and a mixture of *L. plantarum* and *P. acidipropionici* on microorganism counts, chemical composition, fermentative profile, total losses, and aerobic stability of SS.

MATERIALS AND METHODS

This experiment was carried out between January and April 2016 at the Department of Animal Science of Federal University of Grande Dourados, located at 22°14′S, 54°49′W and 450 m of altitude.

Harvesting, Treatments, and Ensiling

Soybeans (*Glycine max* cultivar GMX Cancheiro RR; GMX Genética, Passo Fundo, Brazil) were grown in an experimental field divided in 10 locations within a 4-ha plot until reaching the R6 stage at 105 d (Coffey

Table 1. Chemical composition of whole-plant soybeans before ensiling (g/kg of DM, unless otherwise stated)

Item	Composition
DM, g/kg of fresh material	342
OM	902
NDF	533
ADF	316
CP	195
NFC	121
Ash	97.9
Lignin	86.5
Fat	53.3
NE_L,[1] Mcal/kg	1.88
Buffering capacity, mEq/kg of DM	505

[1]Calculated according to the NRC (2001).

et al., 1995). Approximately 100 kg of soybeans from each location was manually harvested (ground level) and chopped to a theoretical cut of 10 mm using a stationary cutter. 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). Nonfiber 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). Net energy of lactation was estimated according to NRC (2001). Silage buffering capacity was analyzed according to Playne and McDonald (1966).

Four treatments in a factorial arrangement were randomly assigned to 40 experimental silos (plastic buckets, 30 cm in height, and 30 cm in diameter) equipped with Bunsen valves. Two kilograms of sand was placed in the bottom of the buckets and covered with a nylon mesh screen (500 μm) to drain effluents. Inoculant and chitosan were applied individually on the forage assigned for each bucket to generate true replications. Forage was added to the buckets at a compaction rate of 650 kg/m³ and silos were sealed, weighed, and stored at room temperature (25.3 ± 1.8°C; mean ± SD) for 100 d. Treatments consisted of no additives (control; **CON**), 8 g/t of fresh forage microbial inoculant (**INO**; Kera SIL grão úmido, Kera Nutrição Animal, Bento Gonçalves, Brazil); 5 g/kg of fresh forage chitosan (**CHI**), and CHI + INO. Microbial inoculant was composed of *L. plantarum* (4.0 × 10¹⁰ cfu/g) and *P. acidipropionici* (2.6 × 10¹⁰ cfu/g). Microbial inoculant was diluted in water (2 g/L) and sprayed on the forage. According to manufacturer's information (http://www.kerabrasil.com.br/laminas/Kerasil_Grao_Umido.pdf), CHI presented the following technical specifications: 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 Indústria 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. All silos received the same volume of water and cane molasses (40 g/kg of fresh forage).

Microbiological Quality

Samples (100 g) from the intermediate layer (between 10 to 20 cm of silo height) within each silo were collected at the silo opening (d 100) for microbiological profile determination. Ten grams from samples was 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 and Martínez, 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.

Chemical Composition and In Vitro Degradation

Forage samples (500 g) from each experimental silo were collected to assess DM, OM, NFC, CP, EE, NDF, ADF, lignin, ash, and NE_L, as previously described. Dry matter, CP, 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). 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 3 changes of warm tap water.

Fermentative Profile

Silage juice was extracted from forage samples using a hydraulic press and pH was measured using a digital potentiometer (MB-10, Marte, Santa Rita do Sapucaí, Brazil). Silage juice aliquots (2 mL) were mixed with 1 mL of sulfuric acid (1 N) for determination of ammonia nitrogen concentration through colorimetric method described by Foldager (1977).

Volatile fatty acids, ethanol, and lactic acid concentrations in silage juice were determined at the Department of Applied Chemistry of Federal University of São Carlos (Araras, Brazil) according to the methods described by Rodrigues et al. (2012). 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).

Total Losses and Aerobic Stability

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

Gas losses were determined through the equation

$$GL = \frac{SWE - SWO}{DME \times 100},$$

where GL is gas losses (% DM), SWE is the silo weight at ensiling (kg), SWO is the silo weight at opening (kg), and DME is the forage ensiled on DM basis.

Effluent losses were calculated as

$$EL = \frac{SAA - SAB}{FE \times 1,000},$$

where EL is the effluent production (kg of effluent produced/t of forage ensiled), SAA is the weight of silo assembly after the opening (kg), SAB is the weight of silo assembly before ensiling, and FE is the amount of forage ensiled (kg).

Dry matter recovery was estimated as

$$DMR = \frac{FDM}{IDM} \times 100,$$

where FDM is the forage DM after the silos opening (kg) and IDM is the forage DM before the ensiling (kg).

Aerobic stability was considered as the period (h) in which SS temperature remained less than 1°C above the room temperature (Driehuis et al., 2001). Silage was removed and silos were weighted to determine effluent losses. During the 6-d period of aerobic stability evaluation, silos were maintained at room temperature (26.3 ± 2.21, mean ± SD), and temperature of SS was measured every 8 h after oxygen exposure using an infrared thermometer (MS6530, Wiltronics Research Pty. Ltd., Victoria, Australia). In addition, samples (200 g) from minisilos of each treatment were collected every 24 h to determine DM and pH after silo oxygen exposure (Kung et al., 1984).

Statistical Analysis

Data were analyzed using the PROC MIXED of SAS 9.3. (SAS Inst. Inc., Cary, NC), according to the statistical model

$$Y_{ijk} = \mu + C_i + I_j + C \times I_{ij} + e_{ijk},$$

with $e_{ijk} \approx N\left(0, \sigma_e^2\right)$, where Y_{ijk} is the value of dependent variable, μ is the overall mean, C_i is the fixed effect of chitosan ($i = 1, 2$), I_j is the fixed effect of microbial inoculant ($j = 1, 2$), $C \times I_{ij}$ is an interaction term, e_{ijk} is the residual error, and N stands for Gaussian distribution, and σ_e^2 is the variance associated with silos. Degrees of freedom were corrected by Kenward and Roger (1997) method. When the interaction term was declared significant, dismemberment study was performed using Fisher's protected least significant different means test.

Silage pH and DM content after aerobic exposure were analyzed as repeated measures, according the following statistical model

$$Y_{ijkl} = \mu + C_i + I_j + C \times I_{ij} + \omega_{k:ij} + T_l + T \times C_{li} + T \times I_{lj} + T \times C \times I_{lij} + e_{ijkl},$$

with $\omega_{ijk} \approx N\left(0, \sigma_\omega^2\right)$ and $e_{ijkl} \approx MVN(0, R)$, where Y_{ijkl} is the value of dependent variable, μ is the overall mean, C_i is the fixed effect of chitosan ($i = 1, 2$), I_j is the fixed effect of microbial inoculant ($j = 1, 2$), T_l is the fixed effect of time (days, $l = 1$ to 5), $\omega_{k:ij}$ is the random effect of silo k within ith level of chitosan and jth level of inoculant ($k = 1$ to 40), $C \times I_{ij}$, $T \times C_{li}$, $T \times I_{lj}$, and $T \times C \times I_{lij}$ are interaction terms, e_{ijkl} is the residual error, N stands Gaussian distribution, σ_ω^2 is the variance associated with silos, MVN stands for multivariate normal, and R is the variance-covariance matrix of residuals due to the repeated measurements. Compound symmetry (CS), heterogeneous compound symmetry (CSH), autoregressive (AR), heterogeneous autoregressive (ARH), Toeplitz (TOEP), heterogeneous Toeplitz (TOEPH), factor analytic (FA), Huynh-Felt (HF), unstructured (UN), and variance components (VC) variance and covariance matrixes were evaluated using Bayesian method. Statistical analysis of silage pH and DM content were performed using CSH and ARH matrices, respectively. Significance level was set at 0.05.

RESULTS

Microbiological Quality

Chitosan increased ($P \leq 0.05$) overall bacterial counts in SS, including the counts of lactic acid, aerobic, and anaerobic bacteria (Table 2). Inoculant treatment increased ($P \leq 0.05$) LAB counts. Both CHI and INO individually decreased ($P \leq 0.05$) mold and yeast counts in SS. A CHI × INO interaction effect was detected ($P \leq 0.05$) on total bacteria counts, in which CHI and CHI+INO treatments showed the highest counts of

Table 2. Microorganism counts ($\log_{10}$) of soybean whole-plant silage treated with chitosan and bacterial inoculant

Item	Treatment[1]					P-value		
	CON	CHI	INO	CHI+INO	SEM	CHI	INO	CHI × INO
Lactic bacteria	5.62	7.76	7.18	7.32	0.98	0.032	0.041	0.431
Aerobic bacteria	5.95	7.52	6.53	7.20	0.87	0.032	0.553	0.443
Anaerobic bacteria	6.08	7.69	6.95	7.36	1.12	0.031	0.582	0.609
Total bacteria	6.32[b]	7.91[a]	7.09[ab]	7.59[a]	1.09	0.029	0.543	0.032
Mold and yeast	6.45	4.32	4.26	5.45	0.77	0.012	0.021	0.162

[a,b]Values in the same row with a different superscript differ significantly at $P \leq 0.05$ according to Fisher's protected LSD test.

[1]CON = control, no additives; CHI = chitosan inclusion of 5 g/kg of fresh matter; INO = bacterial inoculant mixture of *Lactobacillus plantarum* (at 4.0×10^{10} cfu/g of fresh matter) and *Propionibacterium acidipropionici* (at 2.6×10^{10} cfu/g); CHI+INO = the combination of chitosan and bacterial inoculant.

Table 3. Chemical composition and in vitro degradation of soybean whole-plant silage treated with chitosan and bacterial inoculant (g/kg of DM, unless otherwise stated)

Item	Treatment[1]				SEM	P-value		
	CON	CHI	INO	CHI+INO		CHI	INO	CHI × INO
DM, g/kg of fresh material	294	303	296	291	0.31	0.800	0.457	0.306
Composition								
OM	937	933	929	932	0.16	0.940	0.199	0.377
NDF	459	547	530	520	1.06	0.050	0.246	0.614
ADF	353	366	320	338	1.06	0.480	0.174	0.913
Lignin	82.5	83.4	84.8	80.8	0.06	0.214	0.895	0.055
CP	226	202	200	202	0.52	0.302	0.211	0.231
NFC	200^a	146^b	142^b	162^{ab}	1.05	0.047	0.034	0.030
Ash	63.2	66.5	70.8	68.0	0.16	0.940	0.199	0.377
Fat	51.4	42.7	52.8	48.1	0.19	0.093	0.386	0.611
NE_L,[2] Mcal/kg of DM	1.70^b	1.81^a	1.82^a	1.77^{ab}	0.02	0.013	0.024	0.021
In vitro degradation[3]								
DM	681^b	729^a	725^a	710^{ab}	1.14	0.032	0.053	0.006
CP	701^b	745^a	749^a	730^{ab}	1.06	0.061	0.022	0.014
NDF	620^b	660^a	662^a	643^{ab}	1.02	0.083	0.021	0.023

[a,b]Values in the same row with a different superscript differ significantly at $P \leq 0.05$ according to Fisher's protected LSD test.

[1]CON = control, no additives; CHI = chitosan inclusion of 5 g/kg of fresh matter; INO = bacterial inoculant mixture of *Lactobacillus plantarum* (at 4.0×10^{10} cfu/g of fresh matter) and *Propionibacterium acidipropionici* (at 2.6×10^{10} cfu/g); CHI+INO = the combination of chitosan and bacterial inoculant.

[2]Calculated according to Weiss et al. (1992) and NRC (2001) at maintenance level.

[3]According to Holden (1999) fermented for 48 h.

total bacteria, INO had intermediate values, and CON presented the lowest values.

Chemical Composition and In Vitro Degradation of DM

Chitosan increased ($P \leq 0.05$) NDF content in SS (Table 3). A CHI × INO interaction effect was observed ($P \leq 0.05$) on SS contents of NFC and NE_L, in which the combination of CHI and INO had higher values of NFC content and lower values of NE_L in comparison with CHI and INO alone. Although CHI and INO alone improved in vitro degradation of DM, CP, and NDF of

SS, their combination had no effect on silage nutrient degradation in comparison with CON.

Fermentative Profile

Chitosan increased ($P \leq 0.01$) NH_3-N ratio and lactate concentration and decreased ($P = 0.05$) ethanol concentration in SS (Table 4). A CHI × INO interaction effect ($P \leq 0.05$) was detected on acetate, propionate, butyrate, isobutyrate, and valerate concentrations in SS. Additives decreased acetate concentration in silage, in which CHI+INO had intermediate values of acetate concentration, and additive treatments alone showed

Table 4. Fermentative profile of soybean whole-plant silage treated with chitosan and bacterial inoculant (g/kg of DM, otherwise stated)

Item	Treatment[1]				SEM	P-value		
	CON	CHI	INO	CHI+INO		CHI	INO	CHI × INO
pH	5.33	5.32	5.69	5.19	0.09	0.324	0.543	0.321
NH_3-N, g/kg of total nitrogen	66.6	91.0	79.9	68.4	0.69	0.004	0.653	0.876
Acetate	0.983^a	0.730^c	0.738^c	0.844^b	0.39	0.014	0.008	0.041
Propionate	0.103^b	0.120^a	0.096^b	0.025^c	0.14	0.004	0.126	0.005
Butyrate	2.38^a	2.09^{ab}	1.81^b	0.79^c	1.68	0.321	0.321	0.006
Isobutyrate	0.042^a	0.050^a	0.044^a	0.012^b	0.04	0.532	0.776	0.012
Valerate	0.172^a	0.115^b	0.111^b	0.041^c	0.11	0.002	0.003	0.032
Ethanol	1.38	1.12	1.14	1.21	0.54	0.032	0.675	0.543
Lactate	5.83	7.38	5.90	6.35	0.28	0.002	0.131	0.761

[a–c]Values in the same row with a different superscript differ significantly at $P \leq 0.05$ according to Fisher's protected LSD test.

[1]CON = control, no additives; CHI = chitosan inclusion of 5 g/kg of fresh matter; INO = bacterial inoculant mixture of *Lactobacillus plantarum* (at 4.0×10^{10} cfu/g of fresh matter) and *Propionibacterium acidipropionici* (at 2.6×10^{10} cfu/g); CHI+INO = the combination of chitosan and bacterial inoculant.

Table 5. Total losses and aerobic stability (6-d period) of soybean whole-plant silage treated with chitosan and bacterial inoculant

Item	Treatment[1]				SEM	P-value		
	CON	CHI	INO	CHI+INO		CHI	INO	CHI × INO
Losses								
Gas, g/kg of fresh material	19.4[ab]	21.9[a]	24.5[a]	16.4[b]	0.10	0.159	0.908	0.011
Gas, g/kg of DM	130	108	129	137	0.91	0.002	0.452	0.426
Effluent, kg/t of fresh material	63.8	56.4	66.8	47.0	3.14	0.034	0.594	0.305
Effluent, g/kg of DM	57.5	52.0	61.2	41.8	0.28	0.035	0.547	0.217
Total, % DM	188	160	190	179	0.95	0.007	0.577	0.674
DM recovery, % DM	812	840	810	821	0.95	0.007	0.577	0.674
Aerobic stability, °C								
Accumulated temperature	311	305	311	299	2.28	0.065	0.483	0.506
Maximum	24.8	24.8	25.3	23.9	0.45	0.447	0.853	0.478
Stability	22.8	23.4	22.9	24.4	0.28	0.084	0.364	0.425
Stability, h	81.0	78.0	95.0	96.0	0.35	0.543	0.005	0.641

[a,b]Values in the same row with a different superscript differ significantly at $P \leq 0.05$ according to Fisher's protected LSD test.

[1]CON = control, no additives; CHI = chitosan inclusion of 5 g/kg of fresh matter; INO = bacterial inoculant mixture of *Lactobacillus plantarum* (at 4.0×10^{10} cfu/g of fresh matter) and *Propionibacterium acidipropionici* (at 2.6×10^{10} cfu/g); CHI+INO = the combination of chitosan and bacterial inoculant.

the lowest values of acetate concentration in silage. In general, CHI+INO exhibited the lowest values of propionate, butyrate, isobutyrate, and valerate concentrations in silage.

Total Losses and Aerobic Stability

Chitosan decreased ($P \leq 0.05$) the losses via gas (g/kg of DM) and effluents, and consequently decreased ($P = 0.007$) total losses and increased ($P \leq 0.01$) DMR of SS (Table 5). Although INO had no effect on total losses of SS, INO increased ($P \leq 0.01$) the time in which silage remained stable after oxygen exposure. A CHI × INO interaction effect ($P \leq 0.05$) was observed on gas losses (g/kg of fresh matter) in SS, wherein the lowest value was reported when silage was inoculated with CHI+INO and the highest values were found when silage was treated with CHI or INO.

The DM content of SS constantly increased ($P \leq 0.01$) during the 6-d period after silage exposure to oxygen (Figure 1). Soybean silage pH constantly increased ($P \leq 0.01$) after 6 d of oxygen exposure, but the CHI+INO treatment maintained ($P \leq 0.01$) pH values below 5 until 72 h after the silos opening whereas other treatments exhibited pH above 5 after 24 h of oxygen exposure (Figure 2).

DISCUSSION

In general, the combination of CHI and INO did not demonstrate positive synergistic effects on microbial quality, fermentation profile, and total losses of SS; thus, our hypothesis was not supported by the results. However, CHI increased LAB counts and lactic acid concentration of SS while decreasing its amounts of

mold and yeast. In addition, chitosan-treated silages had the highest values of propionate concentration and DMR. On the other hand, INO only increased silage aerobic stability, with no effects on its fermentative profile. The results above do not support the possible inhibition of INO activity by CHI incorporation in SS, whereas the combination of CHI and INO had minor positive effects (such as higher values of counts of total bacteria and acetate, and lower values of gas losses) on SS quality. Chitosan could exert a negative effect on INO because it has greater bactericidal effects against gram-positive (e.g., *L. plantarum* and *P. acidipropionici*) than gram-negative bacteria, and this effect is enhanced at low pH (Senel and McClure, 2004).

The antifungal property of chitosan (Olicón-Hernández et al., 2015) was confirmed in this experiment. Chitosan suppresses sporulation and spore germination of fungi (Hernández-Lauzardo et al., 2008). Mold and yeasts are undesirable microorganisms that cause silage deterioration, and their inhibition is a result of anaerobiosis and acidification (especially by lactic acid) of ensiled forage (Pahlow et al., 2003). Decreasing the amounts of mold and yeast likely increases the nutrient availability for bacterial growth in SS. In addition, lower counts of mold and yeast also increase oxygen concentration in silage favoring the aerobic bacteria growth, including acetic acid bacteria. We expected that the combination of CHI and INO would exert a positive associative effect in terms of antifungal activity on SS, as chitosan increased lactic acid concentration and decreased the fungi and yeast counts of sugarcane silage (Gandra et al., 2016a) whereas *P. acidipropionici* (present in INO) can metabolize lactic acid into acetate that inhibits yeasts and molds (Moon, 1983). Indeed, the combination of CHI and INO had higher acetate
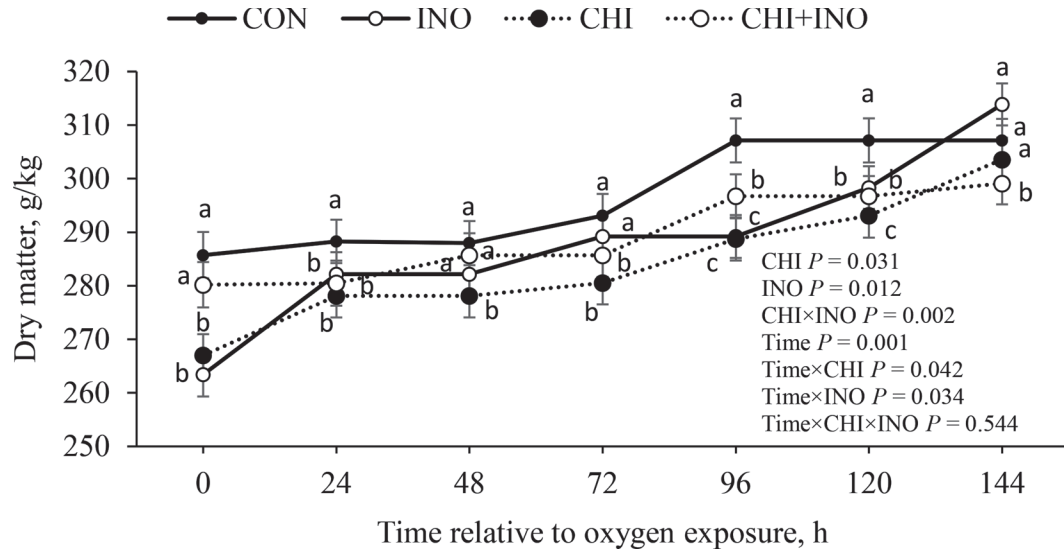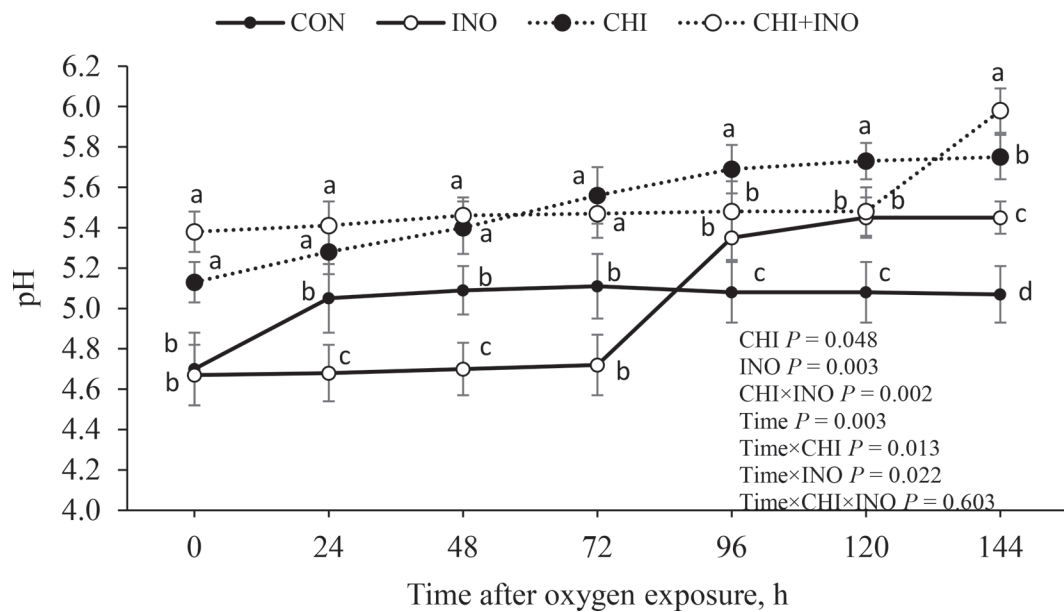
Figure 1. Dry matter content of soybean whole-plant silage treated with chitosan (CHI) or bacterial inoculant (INO) after oxygen exposure. CON = control, no additives; CHI = chitosan inclusion of 5 g/kg of fresh matter; INO = bacterial inoculant mixture of *Lactobacillus plantarum* (at 4.0×10^{10} cfu/g of fresh matter) and *Propionibacterium acidipropionici* (at 2.6×10^{10} cfu/g); CHI+INO = the combination of chitosan and bacterial inoculant. Pooled SEM = 4.04. Error bars represent SE. Letters (a–c) within the same timepoint show that values differed statistically ($P < 0.05$).

concentration but had no effect on mold and yeast counts in comparison with INO alone.

In the current experiment, bacteria showed less sensitivity to the antimicrobial action of chitosan than observed in fungi, agreeing with a review by Kong et al. (2010). It is known that the chitosan bactericidal effect is dependent on pH, with greater activity at pH values around 4.5 (Senel and McClure, 2004). Although the reasons of increased LAB in chitosan-treated silage are not clear, this effect has been previously reported in sugarcane silage treated with chitosan (Gandra et al., 2016a; Del Valle et al., 2018). Agreeing with the current

Figure 2. Soybean whole-plant silage pH treated with chitosan (CHI) or bacterial inoculant (INO) after oxygen exposure. CON = control, no additives; CHI = chitosan inclusion of 5 g/kg of fresh matter; INO = bacterial inoculant mixture of *Lactobacillus plantarum* (at 4.0×10^{10} cfu/g of fresh matter) and *Propionibacterium acidipropionici* (at 2.6×10^{10} cfu/g); CHI+INO = the combination of chitosan and bacterial inoculant. Pooled SEM = 0.17. Error bars represent SE. Letters (a–d) within the same timepoint show that values differed statistically ($P < 0.05$).

study, Gandra et al. (2016a) observed a reduction in fungi counts and ethanol concentration in sugarcane silage. Finally, it is expected that adding LAB to SS would increase LAB counts. An inoculation rate of 10^5 to 10^6 microorganisms per gram of fresh forage is sufficient for inoculated LAB overwhelm the epiphytic flora and become the predominant population in silage (Kung et al., 2003).

Chitosan and inoculant decreased NFC in SS, whereas CHI and INO combination had no synergistic effect on silage composition. The highest values of gas losses (g/kg of fresh material) for CHI- and INO-treated silages suggest a greater fermentation of NFC for these treatments, as NFC are the most available carbohydrate source to be fermented in silos. In addition, the lowest values of NFC were related to the highest values of NDF in SS treated with CHI or INO. Both silage additives increased after 48 h of in vitro degradation of DM, CP, and NDF, as this effect is related to the greater counts of bacteria in silo in comparison with CON. Improvements in NDF degradation are likely related to LAB production of ferulic acid esterase during the silage storage (Nsereko et al., 2008). Ferulic acid esterase acts on ferulic acid cross-linking of xylans to lignin that limits enzymatic degradation of cell wall (Grabber, 2005). Other authors have reported an increase in NDF degradation when crops were ensiled with LAB (Weinberg et al., 2007; Gandra et al., 2017). Furthermore, chitosan addition to sugarcane silage improved DM (1.5%) and NDF (+4.6%) in vitro degradation (Gandra et al., 2016a) and chitosan dietary supplementation promoted DM and CP digestibility in dairy heifers, beef steers, and lactating cows (Araújo et al., 2015; Gandra et al., 2016b; Vendramini et al., 2016).

Chitosan increased the NH_3-N content in SS juice, and this outcome was previously described when adding chitosan to silages (Gandra et al., 2016a). Increased NH_3-N content in SS was somewhat expected, as CP content in chitosan may reach up to 10.8% and it has been used as a nitrogen source for ruminants (Manni et al., 2010; Fadel El-Seed et al., 2013). Furthermore, glucosamine units ($-NH_2$) of chitosan are converted into soluble protonated form ($-NH^+_3$) when chitosan is present in aqueous acid solution (Goy et al., 2009). In general, the combination of CHI and INO jeopardized fermentation profile of SS in relation to CON, decreasing the production of organic acids critical for silage preservation and animal performance, such as acetate and propionate. To our knowledge, no study has evaluated the combination of CHI and INO on SS fermentation, and the reasons for this antagonistic effect needs elucidation.

In the current experiment, CHI improved DMR of SS due to a reduction in gas and effluent losses. Chitosan has decreased gas and effluent losses and increased DMR in sugarcane silage (Gandra et al., 2016a; Del Valle et al., 2018). Several factors can influence the aerobic deterioration or stability in silages after opening a silo, such as the concentrations of DM, acetic acid, and butyric acid and counts of yeast and molds. A positive association exists between aerobic stability and acetic and butyric acids concentration, whereas DM content and yeast are negatively correlated with aerobic stability and DMR (Ohyama et al., 1980). However, we did not find increases in butyric acid and acetic acid production, and INO decreased the counts of mold and yeast in SS. The combination of LAB and propionic acid bacteria has improved the aerobic stability of silages (Zhang et al., 2015). Furthermore, Nkosi et al. (2016b) reported an increase in the aerobic stability of SS treated with mixture of microbial inoculant (*Pediococcus acidilactici* and *Lactobacillus buchneri*) and enzymes (cellulase and hemicellulase).

Greater gas losses (g/kg of natural matter) in the silos treated with CHI or INO alone were likely related to the microbial count results. According to Oliveira et al. (2018), LAB inoculant improves legume silage fermentation with positive effects on DMR and chemical composition. In the present study, besides increased gas losses (probably associated with increased microbial count) silos treated only with CHI had greater DM in vitro degradation. On the other hand, INO had no effect on DMR but increased microbial counts and nutrient in vitro degradation. Furthermore, CHI and INO had no synergistic effect on previously mentioned parameters.

CONCLUSIONS

Both CHI and INO alone increased LAB counts and in vitro degradation of nutrients and reduced counts of mold and yeasts of SS. In addition, CHI increased DMR of SS. The combination of CHI and INO had small positive effects of SS fermentation in comparison with ensiling with CHI or INO alone, such as greater acetate concentration and lower butyrate concentration and gas losses; however, these effects were not associated with decreases in mold and yeast counts and improvements in chemical composition of SS. Therefore, incorporating CHI or INO alone to SS is a suitable form to improve SS nutritive and fermentative quality.

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