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Effect of Chemical and Microbial Additives on Fermentation Profile, Chemical Composition, and Microbial Populations of Whole-Plant Soybean Silage

Jefferson Rodrigues Gandra [1,*], Caio Seiti Takiya [2], Tiago Antonio Del Valle [3], Cibeli de Almeida Pedrini [4], Erika Rosendo de Sena Gandra [5], Giovani Antônio [4], Euclides Reuter de Oliveira [4], Igor Kieling Severo [2] and Francisco Palma Rennó [6,*]

1 Faculdade de Agronomia, Instituto de Estudo em Desenvolvimento Agrário e Regional, Universidade Federal do Sul e Sudeste do Para, Marabá 68507-590, Brazil
2 Academic Department of Agrarian Sciences, Federal University of Technology—Paraná, Via do Conhecimento, Km 1, Pato Branco 85503-390, Brazil; caiotakiya@utfpr.edu.br (C.S.T.); igorsevero@utfpr.edu.br (I.K.S.)
3 Department of Animal Science, Rural Sciences Center, Universidade Federal de Santa Maria—UFSM, Santa Maria 97105-900, Brazil; tiago.valle@ufsm.br
4 Faculdade de Ciências Agrarias, Universidade Federal da Grande Dourados, Dourados 79804-970, Brazil; cibeli_almeida@hotmail.com (C.d.A.P.); giovani_antonio@outlook.com (G.A.); euclidesoliveira@ufgd.edu.br (E.R.d.O.)
5 Faculdade de Zootecnia, Instituto de Estudos no Trópico Úmido, Universidade Federal do Sul e Sudeste do Pará, Marabá 68507-590, Brazil; erikagandra@unifesspa.edu.br
6 Department of Animal Production and Animal Nutrition, University of São Paulo, Pirassununga 13635-900, Brazil
* Correspondence: jeffersongandra@unifesspa.edu.br (J.R.G.); francisco.renno@usp.br (F.P.R.)

Abstract: This study evaluated the effects of two chemical additives or a microbial inoculant on chemical composition and DM losses in whole-plant soybean silage. One-hundred and twenty mini-silos were used in a completely randomized design experiment with the following treatments: water without chloride (control, CON); a microbial inoculant (INO); a chemical additive containing 35–45% formic acid (FA type); and another chemical additive containing 50–60% propionic acid (PA type). Data were analyzed using mixed models of SAS, and treatment differences were evaluated by the following orthogonal contrasts: C1 = CON vs. additives (INO + FA type + PA type); C2 = INO vs. chemical additives (FA type + PA type); and C3 = PA type vs. FA type. Silage pH and ammonia nitrogen concentration were decreased, and concentrations of lactic acid and acetic acid were increased with additives. Counts of lactic acid bacteria were higher in silages with INO than with chemical additives. DM recovery increased with FA type and PA type. Additives increased DM and CP concentrations. Silage A-fraction proportion was greater with additives. Additives, particularly FA type and PA type, improved chemical composition and fermentative profile and reduced undigestible proportions of protein in whole-plant soybean silage. Chemical additives were more effective in reducing silage DM losses than INO.

Keywords: acidification; additives; degradation; organic acid; protein fraction

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1. Introduction

Soybean (*Glycine max*) is currently one of the most important crops worldwide [1], and soybean meal, as its coproduct has been considered the most economical plant protein in animal diets. Whole-plant soybeans (WPSs, including stems, leaves, and fruits) are also easy to produce on a large scale. However, WPS use in animal feeding has faced logistical and nutritional challenges.

Ensiling is commonly used to store forage biomass and maintain nutritional value by fermentation and acid production [2]. After the aerobic stage of ensiling, anaerobic

bacteria (mainly lactic acid bacteria—LAB) produce organic acids, reduce silage pH, and inhibit the growth of spoilage microorganisms [3]. However, legume silages such as WPSs have low water-soluble carbohydrate (WSC) content, insufficient epiphytic LAB count, and high buffer capacity [4]. These conditions favor undesirable fermentation (clostridial and enterobacterial) and reduce silage quality [5].

Several studies evaluated microbial inoculant effects on WPS ensiling [6,7]. However, the delayed effect of microbial inoculant could not be sufficient to improve legume silage fermentation. Therefore, acid addition during the legume ensiling could be a promising strategy in ensiling for these materials [8]. Formic acid is a well-known ensiling additive for low fermentable materials [9]. Sodium propionate has been successfully utilized as a silage additive with a 0.35 benzoate equivalent [10]. In addition, glycerol can be used as an LAB substrate [11]. Wei et al. [12] reported positive effects of formic acid and lactic acid inoculation in different crop silages. However, to the best of our knowledge, there is no study evaluating blends containing formic acid, sodium propionate, glycerol propionate, and glycerol with LAB inoculation instead of WPS ensiling. Therefore, we hypothesized that chemical and microbial additives could reduce fermentation losses, and chemical additives could improve nutritive value of WPS silage compared to LAB inoculation. The present study aimed to evaluate the effects of two chemical additives or a microbial inoculant on WPS silage fermentative profiles, microorganism counts, fermentation losses, chemical compositions, and protein degradability.

2. Materials and Methods

This study was conducted between February and December 2019 at the Department of Animal Sciences of Federal University of Grande Dourados, Mato Grosso do Sul, Brazil (450 m asl': 22°14′ S latitude, 54°49′ W).

2.1. Experimental Design and Treatments

One-hundred and twenty experimental silos were used in a completely randomized design with the following treatments: water without chloride (control, CON); a microbial inoculant (INO) containing 4.0×10^{10} cfu/g *Lactiplantibacillus plantarum* and 2.6×10^{10} cfu/g *Propionibacterium acidipropionici* (Kera SIL grão úmido; Kera Nutrição Animal, Bento Gonçalves, Brazil); a chemical additive containing 35–45% formic acid (ProMyr TMR Flexible EN; Perstorp Waspik BV, Waspik, The Netherlands) added at 2 mL/kg fresh material (FA type); and another chemical additive containing 50–60% propionic acid added (ProMyr TMR Performance, Perstorp Waspik BV) at 2 mL kg/fresh material (PA type). The FA type contained 35–45% formic acid, 15–25% propionic acid, and 10–20% sodium formate, whereas the PA type had 50–60% propionic acid, 15–25% hexanoic acid, 1–5% sodium formate, 1–5% propanetriol, and 15–25% glycerol propionate. The INO was diluted in water without chloride (2 g/L) and sprayed onto the forage. Chemical additives were sprayed onto the forage and hand mixed.

Whole-plant soybeans (*Glycine max*, cultivar GMX Cancheiro RR; GMX Genética, Passo Fundo, Brazil) were harvested at the R7 phenological stage (beginning of maturity; [13]) using a pull-type forage harvester (Sahara 120; HaramaQ, Sertão, Brazil) adjusted to make a theoretical cut of 10 mm from a 0.50 ha area under the crop conditions of Mato Grosso do Sul State. Whole-plant soybeans were planted with 50 cm row spacing. Experimental silos were made in plastic buckets (30 cm height and 30 cm internal diameter) with sand (2 kg) placed in the bottom of buckets separated from the ensiled material by a nylon mesh (500 μm) for posterior measurements of effluent production. Freshly chopped whole-soybean plant material was weighed, and treatments were individually provided before the manual compaction at 500 kg/m^3 specific density. Buckets were equipped with Bunsen valves to avoid gas penetration and allow gas scape and were stored at room temperature for different periods (30, 60, 90, 120, 150, or 180 d). Samples (n = 4) of the ensiled material were collected for chemical analysis (Table 1). Samples were analyzed for contents of DM (method 950.15), ash (method 942.05), CP (N × 6.25; method 984.13;

Kjeldahl), and ether extract (EE; method 920.39) according to AOAC [14]. Neutral detergent fiber (aNDF) with heat-stable alpha-amylase [15] and sodium sulfite, ADF (TE-149 fiber analyzer, Tecnal Laboratory Equipment Inc., Piracicaba, Brazil), and lignin (sulfuric acid method) was determined according to Van Soest et al. [16] and AOAC [14]. The residues of NDF and ADF analyses were also submitted to N analysis to determine neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) concentrations. Total digestible nutrient and net energy of lactation were calculated according to NRC [17]. Non-fiber carbohydrate (g/kg) was calculated as = 1000 − (aNDF + CP + EE + ash), all values expressed as g/kg. Protein fractions were evaluated according to Licitra et al. [18]. Briefly, non-protein N (A-fraction) was analyzed using trichloroacetic acid. Soluble protein (B1) was analysed after borofosphate solubilization. The acid detergent insoluble N (fraction C) and NDIN were obtained analyzing N content of residual fiber, as previously described. The B3 fraction was obtained by NDIN and C-fraction difference. The B2 fraction was obtained by the difference between the total N and described fractions.

Table 1. Chemical composition and effective degradability of whole-plant soybean before ensiling.

Item (% DM)	Mean	SD
Chemical		
Dry matter	36.7	0.23
Organic matter	93.6	0.13
Crude protein	16.5	0.02
Neutral detergent insoluble nitrogen	4.44	0.56
Acid detergent insoluble nitrogen	2.73	0.04
Ether extract	7.90	0.15
Neutral detergent fiber	39.4	0.43
Acid detergent fiber	29.9	0.19
Lignin	7.06	0.69
Non-fiber carbohydrate	29.6	0.23
Total digestible nutrient	67.5	0.36
NEL (Mcal/kg) [1]	1.53	0.02
Degradation		
A-fraction [2]	314	45.4
B-fraction [3]	398	40.1
C-fraction [4]	288	23.5
Kdb (g/kg/h) [5]	92.9	9.74
Effective degradability [6]		
20	641	29.2
50	572	35.9
80	527	39.6

[1] Calculated according to NRC (2001). [2] Soluble fraction. [3] Potentially degradable fraction. [4] Non-degradable fraction. [5] Degradation rate of B-fraction (g/kg/h). [6] Effective degradability (g/kg DM) considering passage rates of 20, 50, and 80 g/kg/h.

2.2. Chemical Compositions, Fermentative Profiles, and Microorganism Counts

Samples from 3 experimental silos per treatment of each storage length were submitted to chemical analysis for determination of DM, organic matter, CP, NDF, ADF, NIDN, NIDA, ether extract, and lignin, as described earlier. One sample from each silo was prepared for pH measures according to Kung Jr. et al. [19] whereas pH was measured by a pH electrode. One sample from each silo was analyzed for concentrations of ammonia nitrogen (NH_3-N) according to Chaney and Marbach [20]. One sample from each experimental silo was also analyzed for concentrations of organic acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid) according to Erwin et al. [21]. The lactic acid concentration of silages was determined by a colorimetric method proposed by Pryce [22].

Samples of silage (10 g, n = 5 per treatment of each storage length) were diluted into sterilized sodium chloride solution (9 g/L, 90 mL). Microorganism counting was carried

out in triplicate through decimal dilution series (10^{-1} to 10^{-6}) in plates with MRS agar (De Man, Rogosa and Sharpe; Granucult prime, Sigma-Aldrich, São Paulo, Brazil) for LAB culture [23], nutrient agar for culture of anaerobic bacteria (48 h incubation period at 37 °C), and counts of yeast and mold were performed according to Rabie et al. [24], using potato dextrose agar and incubation for 120 h at 26 °C. The absolute values were obtained from cfu and then log transformed.

2.3. Dry Matter Losses

Fermentation losses were measured as gas losses (GLs), effluent losses (ELs), and total losses (TLs). After 30, 60, 90, 120, 150, or 180 d of ensiling, experimental silos were weighed to determine gas losses through the following equation [25]:

$$\mathrm{GL}\left(\frac{\mathrm{g}}{\mathrm{kg}}\right) = \frac{\mathrm{FSWe\ (g)} - \mathrm{FSWo\ (g)}}{\mathrm{DMe\ (kg)}},$$

where FSWe is the experimental silo weight at ensiling (g), FSWo is the full experimental silo weight at silo opening (g), and DMe is the dry matter (kg) at ensiling.

Effluent losses were determined through the following equation [25]:

$$\mathrm{EL}\left(\frac{\mathrm{g}}{\mathrm{kg}}\right) = \frac{\mathrm{ESWo\ (g)} - \mathrm{ESWe\ (g)}}{\mathrm{DMe\ (kg)}},$$

where ESWo is the empty experimental silo weight at opening (g), and ESWe is the empty experimental silo at ensiling (g). Total losses were determined by the sum of GL and EL [25].

Dry matter recovery was calculated as the ratio between DM at silo opening (DMo; kg) and DMe (kg) [25]:

$$\mathrm{DMR}\left(\frac{\mathrm{g}}{\mathrm{kg}}\right) = \frac{\mathrm{DMo\ (kg)}}{\mathrm{DMe\ (kg)}} \times 1000$$

2.4. In Situ Degradability

Samples of fresh material (n = 5) and silages from different treatments (n = 5 from each storage length) were dried in forced ventilation oven at 65 °C for 72 h and ground in knives rotary mill to pass through a 2 mm screen. Ground samples (0.5 g) were placed in non-woven tissue bags (5 × 5 cm, 25 µm porosity, and 100 g/m^2; DKN NonWoven TNT, Caçador, Brazil) as described by Casali et al. [26]. Bags with samples were incubated in the rumen of three ruminal cannulated crossbred cows fed a diet composed of 60% of corn silage and 40% concentrate. Samples were incubated in triplicate by storage length, treatment, animal, and incubation period. Samples were removed from the rumen after 2, 4, 8, 16, 24, 48, and 72 h of incubation and washed in running tap water until the water rinsed clear. The same procedure was applied to samples not incubated in the rumen to represent 0 h of incubation. Bags were then dried in a forced ventilation oven for 72 h at 65 °C and analyzed for DM, as previously described.

2.5. Statistical Analysis

Data were analyzed using SAS software (version 9.1.3; SAS Institute, Cary, NC, USA). Data were analyzed for normal distribution of residues using the UNIVARIATE procedure of SAS. Then, data were analyzed as repeated measures using the MIXED procedure of SAS using the following model:

$$Y_{ij} = \mu + A_i + T_j + AT_{jj} + e_{ij}$$

where μ = overall mean, A_i = the fixed of ith treatment, T_j = the fixed effect of jth time, $A_j * T_j$ = the interaction effect between treatment and time, and e_{ij} = residue. Differences among treatments were evaluated by orthogonal contrasts to test the effect of additives

(C1 = CON vs. INO + FA type + PA type), the type of additive (C2 = INO vs. FA type + PA type), or the chemical additive formulation (C3 = PA type vs. FA type). Significance was declared when $p \leq 0.05$.

3. Results

Silage pH and NH_3-N concentration were lower ($p \leq 0.021$) and concentrations of lactic acid, acetic acid, butyric acid, valeric acid, isovaleric acid, and branched-chain fatty acids (BCFAs) were greater ($p \leq 0.031$) in ensiled material with additives than CON (Table 2). Silage pH and concentrations of NH_3-N, ethanol, acetic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and BCFA were greater ($p \leq 0.016$) in ensiled material with INO than with chemical additives (FA type and PA type). Comparisons between chemical additives revealed lower ($p \leq 0.012$) pH and concentrations of propionic acid, isobutyric acid, isovaleric acid, and BCFA in ensiled material with PA type than FA type. Counts of LAB, anaerobic bacteria, and mold and yeast were lower ($p < 0.001$), and count of aerobic bacteria was higher ($p < 0.001$), in silages with additives than CON. Counts of LAB and anaerobic bacteria were higher ($p < 0.001$) and counts of mold and yeast were lower ($p < 0.001$) in silages with INO than with chemical additives. Comparisons between chemical additives revealed greater ($p < 0.001$) counts of LAB and anaerobic bacteria and lower ($p < 0.001$) counts of yeast and mold in silage with PA in comparison to those with FA.

Table 2. Fermentative profile and microorganism count of whole-plant soybean silage treated with microbial inoculant or chemical additives.

Item	Treatment [1]				SEM	*p*-Value [2]					
	CON	INO	FA Type	PA Type		Trt	Time	Trt × Time	C1	C2	C3
pH	5.38	5.54	4.33	4.74	0.053	<0.0001	0.039	0.002	<0.0001	<0.0001	<0.0001
NH_3-N (g/kg total N)	43.7	45.6	30.5	32.7	2.658	0.002	<0.0001	0.001	0.021	<0.0001	0.578
Ethanol (g/kg DM)	0.897	1.00	0.846	0.812	0.039	0.005	<0.0001	<0.0001	0.783	<0.0001	0.468
Lactic acid (g/kg DM)	9.90	11.8	12.6	12.4	0.767	0.021	0.421	0.254	0.001	0.214	0.547
Acetic acid (g/kg DM)	3.85	3.90	3.17	3.03	0.091	<0.0001	<0.0001	<0.0001	0.009	<0.0001	0.422
Propionic acid (g/kg DM)	0.991	1.15	0.977	1.17	0.037	0.008	<0.0001	<0.0001	0.063	0.205	0.007
Butyric acid (g/kg DM)	6.68	9.26	7.31	8.16	0.660	0.006	<0.0001	<0.0001	0.003	0.006	0.184
Isobutyric acid (g/kg DM)	0.427	0.523	0.394	0.465	0.033	0.006	<0.0001	<0.0001	0.196	0.008	0.024
Valeric acid (g/kg DM)	0.694	0.988	0.711	0.865	0.062	<0.0001	<0.0001	0.608	<0.0001	<0.0001	0.167
Isovaleric acid (g/kg DM)	0.694	0.865	0.663	0.841	0.060	0.002	<0.0001	<0.0001	0.031	0.016	0.001
BCFA [3] (g/kg DM)	1.81	2.37	1.76	2.17	0.152	0.004	<0.0001	<0.0001	0.027	0.003	0.012
Microorganism counts (log_{10})											
Lactic acid bacteria	7.24	7.58	6.38	6.73	0.067	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Anaerobic bacteria	6.27	5.90	4.99	6.43	0.120	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Mold and yeast	5.20	4.92	5.64	3.65	0.201	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1] Control (CON), without silage additives; microbial inoculant (INO), 4 g/ton inclusion of a blend of bacteria (Kera SIL grão úmido; Kera Nutrição Animal, Bento Gonçalves, Brazil) containing 4.0×10^{10} cfu/g *Lactiplantibacillus plantarum* and 2.6×10^{10} cfu/g *Propionibacterium acidipropionici*; formic acid type (FA type), 2 mL/kg fresh material of a chemical additive containing 35–45% formic acid, 15–25% propionic acid, and 10–20% sodium formate (ProMyr TMR Flexible EN; Perstorp Waspik BV, Waspik, The Netherlands); and propionic acid type (PA type), 2 mL/kg fresh material of a chemical additive containing 50–60% propionic acid, 15–25% hexanoic acid, 1–5% sodium formate, 1–5% propanetriol, and 15–25% glycerol propionate (ProMyr TMR Performance, Perstorp Waspik BV). [2] Probabilities for treatment effect (Trt), interaction effect between time and treatment (Trt × time), and orthogonal contrasts: C1 = CON vs. silage additives (INO + FA type + PA type), C2 = INO vs. chemical additives (FA type + PA type), and C3 = FA type vs. PA type. [3] Branched-chain fatty acids (isobutyric acid + valeric acid + isovaleric acid).

Gas losses (% DM) and total losses decreased ($p < 0.001$), and DM recovery increased ($p < 0.001$) when additives were incorporated to the silage, especially FA type and PA type (Table 3). When comparing the type of silage additive (INO vs. chemical additives), gas losses, effluent losses, and total losses were lower ($p \leq 0.039$), and DM recovery was greater

($p < 0.001$) for silages treated with chemical additives in comparison with INO. Gas losses were greater ($p = 0.032$) for PA type than FA type. On the other hand, effluent losses were lower ($p \leq 0.002$) for PA-type silages in comparison with FA type. Dry matter recovery was greater ($p = 0.032$) for FA-type than PA-type silages.

Table 3. Fermentative losses and dry matter (DM) recovery of whole-plant soybean silage treated with microbial inoculant or chemical additives.

Item	Treatment [1]				SEM	*p*-Value [2]					
	CON	INO	FA Type	PA Type		Trt	Time	Trt × Time	C1	C2	C3
Losses											
Gas (% DM)	13.8	16.5	4.96	6.51	0.546	<0.0001	<0.0001	0.008	<0.0001	<0.0001	0.032
Effluent (kg/ton)	9.55	10.8	10.2	6.16	0.436	0.001	<0.0001	0.913	0.562	0.005	0.002
Total (% DM)	14.7	17.4	5.96	7.09	0.556	<0.0001	<0.0001	0.006	<0.0001	<0.0001	0.127
DM recovery (%)	86.2	83.5	95.0	93.5	0.546	<0.0001	<0.0001	0.003	<0.0001	<0.0001	0.032

[1] Control (CON), without silage additives; microbial inoculant (INO), 4 g/ton inclusion of a blend of bacteria (Kera SIL grão úmido; Kera Nutrição Animal, Bento Gonçalves, Brazil) containing 4.0×10^{10} cfu/g *Lactiplantibacillus plantarum* and 2.6×10^{10} cfu/g *Propionibacterium acidipropionici*; formic acid (FA type), 2 mL/kg fresh material of a chemical additive containing 35–45% formic acid, 15–25% propionic acid, and 10–20% sodium formate (ProMyr TMR Flexible EN; Perstorp Waspik BV, Waspik, The Netherlands); and propionic acid (PA type), 2 mL/kg fresh material of a chemical additive containing 50–60% propionic acid, 15–25% hexanoic acid, 1–5% sodium formate, 1–5% propanetriol, and 15–25% glycerol propionate (ProMyr TMR Performance, Perstorp Waspik BV). [2] Probabilities for treatment effect (Trt), interaction effect between time and treatment (Trt × time), and orthogonal contrasts: C1 = CON vs. silage additives (INO + FA type + PA type), C2 = INO vs. chemical additives (FA type + PA type), and C3 = FA type vs. PA type.

In general, incorporating additives to silages increased concentrations of DM, OM, CP, NFC, TDN, and NE_L and decreased concentrations of NDF in comparison to CON (Table 4). Silage concentrations of DM, OM, CP, NDF, ADF, TDN, and NE_L increased ($p \leq 0.019$) by treating silages with chemical additives in comparison to INO. When the effects of chemical additives were contrasted, silages with PA type exhibited greater ($p \leq 0.004$) concentrations of NDF and lower concentrations of ADF, lignin, and NFC than silages with FA type.

Table 4. Chemical composition of whole-soybean silage treated with microbial inoculant or chemical additives (g/kg, unless stated).

Item	Treatment [1]				SEM	*p*-Value [2]					
	CON	INO	FA Type	PA Type		Trt	Time	Trt × Time	C1	C2	C3
Dry matter	332	323	362	355	1.929	<0.0001	<0.0001	0.017	<0.0001	<0.0001	0.214
Organic matter	920	919	929	929	0.606	<0.0001	0.081	0.004	<0.0001	<0.0001	0.518
Crude protein	174	176	192	195	1.788	<0.0001	<0.0001	0.028	<0.0001	<0.0001	0.566
Ether extract	83.6	81.9	81.3	84.6	0.835	0.114	<0.0001	<0.0001	0.405	0.402	0.132
Neutral detergent fiber	435	409	375	410	3.535	<0.0001	0.006	<0.0001	<0.0001	0.019	<0.0001
Acid detergent fiber	278	277	294	282	2.732	0.003	<0.0001	0.001	0.067	0.002	0.004
Lignin	81.5	80.4	86.7	82.6	0.881	0.008	<0.0001	0.008	0.179	0.003	0.010
Non-fiber carbohydrate	227	253	280	240	4.059	<0.0001	<0.0001	0.595	<0.0001	0.319	<0.0001
Total digestible nutrient	653	657	671	667	1.290	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.085
NE_L [3] (Mcal/kg)	1.58	1.59	1.63	1.62	0.003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.095

[1] Control (CON), without silage additives; microbial inoculant (INO), 4 g/ton inclusion of a blend of bacteria (Kera SIL grão úmido; Kera Nutrição Animal, Bento Gonçalves, Brazil) containing 4.0×10^{10} cfu/g *Lactiplantibacillus plantarum* and 2.6×10^{10} cfu/g *Propionibacterium acidipropionici*; formic acid (FA type), 2 mL/kg fresh material of a chemical additive containing 35–45% formic acid, 15–25% propionic acid, and 10–20% sodium formate (ProMyr TMR Flexible EN; Perstorp Waspik BV, Waspik, The Netherlands); and propionic acid (PA type), 2 mL/kg fresh material of a chemical additive containing 50–60% propionic acid, 15–25% hexanoic acid, 1–5% sodium formate, 1–5% propanetriol, and 15–25% glycerol propionate (ProMyr TMR Performance, Perstorp Waspik BV). [2] Probabilities for treatment effect (Trt), interaction effect between time and treatment (Trt × time), and orthogonal contrasts: C1 = CON vs. silage additives (INO + FA type + PA type), C2 = INO vs. chemical additives (FA type + PA type), and C3 = FA type vs. PA type. [3] Net energy of lactation.

Greater ($p = 0.006$) proportions of A-fraction and lower ($p = 0.002$) proportions of C-fraction were observed in silages treated with additives than CON (Table 5). The effective degradability of silages was greater ($p < 0.001$) in those treated with additives regardless

of the passage rate used for calculations. Proportions of A-fraction and degradation rate of B-fraction were greater ($p \leq 0.034$), and proportions of B- and C-fractions were lower ($p < 0.001$) in silages treated with chemical additives in comparison with INO. Effective degradability was greater ($p < 0.001$) in silages treated with chemical additives in comparison with INO, regardless of passage rate used in calculations. Degradation rate of B-fraction was greater ($p < 0.001$) for silages treated with PA type than FA type. Effective degradability of DM using passage rates of 50 and 80 g/kg/h in calculations was greater ($p \leq 0.012$) for silages with FA type than PA type.

Table 5. Protein fractions and effective degradability of whole-plant soybean silage treated with microbial inoculant or chemical additives.

Item	Treatment [1]				Storage Length (d)						SEM	*p*-Value [2]								
	CON	INO	FA Type	PA Type	30	60	90	120	150	180		Trt	Time	Trt × Time	C1	C2	C3	C1 × Time	C2 × time	C3 × Time
Protein fractions (g/kg CP) [3]																				
A-fraction	274	252	345	367	326	326	296	315	305	325	34.0	<0.001	0.587	0.093	0.006	<0.001	0.273	0.064	0.110	0.537
B-fraction	354	379	328	318	355	357	333	351	338	336	6.7	<0.001	0.623	0.273	0.301	<0.001	0.441	0.489	0.089	0.558
C-fraction	372	369	326	315	320	348	352	359	357	338	30.1	<0.001	0.164	0.028	0.002	<0.001	0.401	0.058	0.023	0.390
Kdb [4] (g/kg/h)	115	97	298	112	180	215	133	115	150	138	32.4	0.002	0.743	0.980	0.268	0.034	0.002	0.949	0.957	0.643
Effective degradability [5]																				
20	558	560	625	623	625	604	577	582	575	587	26.1	<0.001	<0.001	<0.001	<0.001	<0.001	0.734	0.026	0.001	0.189
50	501	495	585	570	574	555	520	526	521	531	23.1	<0.001	<0.001	<0.001	<0.001	<0.001	0.012	0.002	<0.001	0.276
80	465	453	558	537	540	522	485	489	487	498	22.1	<0.001	<0.001	0.001	<0.001	<0.001	0.001	0.002	0.001	0.580

[1] Control (CON), without silage additives; microbial inoculant (INO), 4 g/ton inclusion of a blend of bacteria (Kera SIL grão úmido; Kera Nutrição Animal, Bento Gonçalves, Brazil) containing 4.0×10^{10} cfu/g *Lactiplantibacillus plantarum* and 2.6×10^{10} cfu/g *Propionibacterium acidipropionici*; formic acid (FA type), 2 mL/kg fresh material of a chemical additive containing 35–45% formic acid, 15–25% propionic acid, and 10–20% sodium formate (ProMyr TMR Flexible EN; Perstorp Waspik BV, Waspik, The Netherlands); and propionic acid (PA type), 2 mL/kg fresh material of a chemical additive containing 50–60% propionic acid, 15–25% hexanoic acid, 1–5% sodium formate, 1–5% propanetriol, and 15–25% glycerol propionate (ProMyr TMR Performance, Perstorp Waspik BV). [2] Probabilities for treatment effect (Trt), interaction effect between time and treatment (Trt × time), and orthogonal contrasts: C1 = CON vs. silage additives (INO + FA type + PA type), C2 = INO vs. chemical additives (FA type + PA type), and C3 = FA type vs. PA type. [3] Soluble fraction (A-fraction), potentially degradable fraction (B-fraction), and non-degradable fraction (C-fraction). [4] Degradation rate of B-fraction. [5] Effective degradability (g/kg DM) considering passage rates of 20, 50, and 80 g/kg/h.

When the effects of silage additives on protein fractions and effective degradability in each storage length were analyzed, silage A-fraction proportion was greater ($p \leq 0.020$) in silos with additives (INO, FA type, and PA type) in comparison with CON after 120 and 150 d of storage (Table 6). Silage A-fraction proportion was greater ($p \leq 0.041$) for silages with chemical additives than INO after 60, 90, 150, and 180 d of storage. B-fraction proportion was lower ($p \leq 0.028$) in silages with chemical additives than INO after 90 and 150 d of storage. C-fraction proportions were lower ($p \leq 0.020$) in silages with additives in comparison with CON after 120 and 150 d of storage. After 120 and 150 d of storage, proportion of C-fraction in silages was lower when additives were applied. C-fraction proportions were greater ($p \leq 0.019$) in silages treated with INO in comparison with those treated with chemical additives after 60, 150, and 180 d of storage. The degradation rate of B-fraction in silages treated with chemical additives was greater ($p = 0.023$) than in those treated with INO. Effective degradability of DM was greater ($p \leq 0.004$) when additives were applied in the silages in all storage lengths, except for 30 d. In general, effective degradability of DM was greater ($p \leq 0.011$) in silages treated with chemical additives in comparison with INO in all storage lengths, except for 30 d storage length and using a passage rate of 20 g/kg/h in estimations. Effective degradability of DM, regardless of feed passage rate, was greater ($p \leq 0.019$) in silages treated with chemical additives in comparison with those treated with INO only after 120 d of storage.

Table 6. Decomposition of protein fractions and effective degradability according to silage additives and storage length of whole-plant soybean silage.

Item/Storage Length	Treatment [1]				*p*-Value [2]			
	CON	INO	FA Type	PA Type	Trt	C1	C2	C3
A								
30	342	312	318	331	0.927	0.586	0.766	0.795
60	272	245	316	351	0.148	0.416	0.041	0.481
90	257	219	358	425	<0.001	0.058	<0.001	0.177
120	219	265	344	334	0.041	0.020	0.083	0.850
150	210	239	408	365	0.001	0.009	0.001	0.381
180	343	231	329	399	0.011	0.554	0.003	0.154
B								
30	345	390	353	330	0.353	0.648	0.109	0.502
60	342	375	365	344	0.719	0.490	0.491	0.540
90	361	415	285	271	<0.001	0.186	<0.001	0.682
120	375	364	346	318	0.380	0.258	0.293	0.406
150	368	372	280	330	0.042	0.222	0.028	0.147
180	333	360	340	312	0.568	0.880	0.256	0.409
C								
30	313	298	329	339	0.600	0.736	0.203	0.746
60	387	380	319	305	0.027	0.054	0.019	0.680
90	382	366	356	304	0.102	0.139	0.203	0.110
120	406	372	310	348	0.032	0.020	0.131	0.245
150	424	389	313	305	0.003	0.007	0.006	0.813
180	324	409	331	289	0.005	0.465	0.001	0.200
KDB								
30	116	91	307	207	0.401	0.453	0.174	0.473
60	163	111	456	129	0.056	0.544	0.139	0.023
90	84	94	301	52	0.273	0.570	0.494	0.079
120	121	88	138	115	0.987	0.951	0.752	0.866
150	166	93	246	97	0.663	0.878	0.518	0.290
180	42	106	337	70	0.146	0.262	0.421	0.061
ED20								
30	626	623	630	621	0.966	0.918	0.888	0.632
60	571	560	639	645	<0.001	0.004	<0.001	0.741
90	544	552	606	608	0.000	0.003	0.001	0.920
120	537	554	640	598	<0.001	<0.001	<0.001	0.019
150	524	542	607	628	<0.001	<0.001	<0.001	0.221
180	549	531	631	638	<0.001	0.001	<0.001	0.693
ED50								
30	570	554	589	582	0.069	0.660	0.011	0.605
60	527	500	600	595	<0.001	0.001	<0.001	0.669
90	478	479	569	554	<0.001	<0.001	<0.001	0.265
120	479	488	589	547	<0.001	<0.001	<0.001	0.003
150	469	477	568	569	<0.001	<0.001	<0.001	0.935
180	484	472	593	576	<0.001	<0.001	<0.001	0.216
ED80								
30	534	512	560	553	0.004	0.483	<0.001	0.628
60	495	460	574	560	<0.001	0.002	<0.001	0.296
90	437	433	544	526	<0.001	<0.001	<0.001	0.192
120	439	447	555	514	<0.001	<0.001	<0.001	0.005
150	432	436	548	533	<0.001	<0.001	<0.001	0.283
180	452	433	566	540	<0.001	<0.001	<0.001	0.060

[1] Control (CON), without silage additives; microbial inoculant (INO), 4 g/ton inclusion of a blend of bacteria (Kera SIL grão úmido; Kera Nutrição Animal, Bento Gonçalves, Brazil) containing 4.0×10^{10} cfu/g *Lactiplantibacillus plantarum* and 2.6×10^{10} cfu/g *Propionibacterium acidipropionici*; formic acid (FA type), 2 mL/kg fresh material of a chemical additive containing 35–45% formic acid, 15–25% propionic acid, and 10–20% sodium formate (ProMyr TMR Flexible EN; Perstorp Waspik BV, Waspik, The Netherlands); and propionic acid (PA type), 2 mL/kg fresh material of a chemical additive containing 50–60% propionic acid, 15–25% hexanoic acid, 1–5% sodium formate, 1–5% propanetriol, and 15–25% glycerol propionate (ProMyr TMR Performance, Perstorp Waspik BV). [2] Probabilities for treatment effect (Trt), interaction effect between time and treatment (Trt × time), and orthogonal contrasts: C1 = CON vs. silage additives (INO + FA type + PA type), C2 = INO vs. chemical additives (FA type + PA type), and C3 = FA type vs. PA type.

4. Discussion

It was hypothesized that silage additives would reduce DM losses and improve chemical composition and fermentation metabolites in WPS silage, whereas chemical additives

would be more effective than INO in improving silage traits due to direct effect in decreasing pH. Indeed, chemical additives reduced silage DM losses, and additives improved chemical composition (i.e., greater CP and lower NDF concentration), increased lactic acid concentration, and reduced C-fraction proportion (undigestible CP) in WPS silage.

To the best of our knowledge, there is no study that compared fermentation traits of WPS ensiled with INO or chemical additives containing formic acid or propionic acid. In whole-plant corn silage, WSC concentration and pH at opening were similar between silage treated with LAB and with organic acids [27]. In a mixed silage (alfalfa and perennial ryegrass), the pH was lower and WSC was greater in silages treated with formic acid regardless of the storage period (7, 15, or 45 d) in comparison with those treated with LAB [28]. Regarding comparisons between chemical additives, silages treated with FA type had greater concentrations of NFC than those silages treated with PA type, aligning with lower silage pH at opening for FA silos in comparison with PA type. Formic acid is a stronger acid than propionic acid because of the inductive effect. Longer hydrocarbon chains exert a greater electron-pushing effect toward the carboxylic group, making the release of proton more difficult. Consequently, the easier the release of a proton, the stronger the acid [29].

Chemical additives reduced NH_3-N content in WPS silage indicating lower proteolysis during silage fermentation, which is further confirmed by the greater concentration of CP observed in these silages when compared to other treatments. Aligning with the current study, authors have reported that chemical additives with organic acids are able to decrease ammonia N content in mixed legume–grass silage [28]. It is important to note that fermentation parameters and metabolites were assessed at the opening of the silos, and they cannot represent the fermentation dynamics throughout the silage storage period. In general, additives increased lactic acid and butyric acid concentration and decreased acetic acid concentration in silage. These results contrast with counts of LAB, which were lowered by FA-type and PA-type treatments. It was anticipated that silages treated with INO would exhibit greater lactic acid content as the additive was composed of LAB. An inoculation rate of 10^5 to 10^6 microorganisms per gram of fresh forage is adequate for inoculated LAB to outcompete the epiphytic flora and establish themselves as the predominant population in silage [30]. Organic acids present in chemical additives, however, should be able to rapidly decrease silage pH, thereby inhibiting bacteria activity and minimizing the production of fermentation metabolites, including lactic and other acids. Aligning with the later statement, lactic acid and anaerobic bacteria counts were decreased in silages treated with FA type. Gheller et al. [31] observed decreased contents of lactic acid and acetic acid in whole-plant corn silage treated with chemical additives containing organic acids. The reasons for the increased silage content of lactic acid observed in the current study are not clear. Mold and yeast counts were decreased by adding silage additives, particularly due to INO and PA type effects. Microbial inoculants combined homofermentative LAB with *P. acidipropionici* to minimize the aerobic spoilage of silages [32]. Propionic acid bacteria metabolize sugars and lactic acid into acetic and propionic acids, compounds known to inhibit the growth of yeast and molds [33]. The effect of INO in reducing counts of mold and yeast of WPS silage was previously reported [34].

During the fermentation phases of silage, DM losses are derived from effluent and gas losses. Before the active fermentation phase, the oxygen trapped in the silo allows biological and chemical processes producing effluent, carbon dioxide, heat, and free NH_3-N [35]. When silo becomes anaerobic, the losses are primarily from carbon dioxide production. The amount of DM loss depends on the dominant microbial species and the substrate fermented [36]. Despite a significant effect on gas losses observed when contrasting CON with all silage additives, only chemical additives reduced losses and increased DM recovery. In addition, PA-type treatment was more effective in reducing effluent losses than FA. It is well known that chemical additives promote a rapid drop in silage pH with concomitant bacteriostatic and fermentation inhibitor effects, thus decreasing nutrient losses of the ensiled herbage [3,37]. The reasons for lower effluent losses in silages treated with PA type

in comparison with FA type might be related to its ability in altering the cell permeability of microorganisms and competing with amino acids for space on active enzyme sites [30]. Furthermore, silages treated with PA type exhibited lower counts of mold and yeast than FA-type-treated silages, which might have reduced aerobic deterioration. The literature lacks data on the effects of chemical additives with organic acids on WPS silage fermentation, but studies have reported improved DM recovery when different additives were included in whole-plant corn silage or in snaplage [31,38]. Agreeing with the current study, authors reported no effect of INO on WPS silage total losses [34]. However, improved DM recovery or decreased total losses when the same microbial inoculant was applied in WPS silage was reported by Morais et al. [7]. Inconsistent results can be related to phenological stage, which soybean plant was harvested (i.e., R6 vs. R7), and DM content of ensiled material (25.0 vs. 36.7% DM) when comparing the results of Morais et al. [7] with those observed in the current study.

Regarding chemical composition, silages with chemical additives exhibited greater concentrations of DM, CP, and NFC, leading to increased NFC and TDN proportions. Silages with additives had lower concentrations of NDF, which is likely associated with lower solubilization or degradation of non-fiber components in comparison with CON. These results, especially differences in DM content, are supported by greater DM recovery of silages treated with chemical additives. Agreeing with the current study, authors reported greater concentrations of OM, CP, and NFC when ensiling whole-plant corn with different organic acid preparations composed mostly of formic acid and propionic acid [31]. Other studies have also shown positive effects on nutritive value of silages treated with chemical additives with organic acids [39,40], even when compared to silages treated with microbial inoculant [41]. Whole-soybean silages treated with INO, however, had lower concentrations of DM, CP, NFC, and TDN in comparison with silages with organic acids. Collectively, these results indicate that chemical additives are more effective in preserving chemical composition of the whole soybean plant. Although we did not measure the decrease in silage pH during the different phases of silage fermentation, it is somewhat expected that chemical additives promoted a rapid drop in silage pH in comparison with INO and thus interrupted the consumption of cellular content of plant cells by microbes faster than INO. In addition, silage pH at silos' openings was lower in silages treated with organic acids than INO.

Concerning protein fractions in WPS silage, chemical additives (FA type and PA type) increased contents of A-fraction and decreased contents of B- and C-fractions, indicating low proteolytic activity in silos. Furthermore, chemical additives also increased digestion rate of B-fraction and effective degradability of CP. It is expected that increasing contents of A-fraction, which is promptly soluble in the rumen, and the digestion rate of the potentially degradable fraction (B-fraction) would increase effective degradability of silage. De Morais et al. [7] reported greater proportions of A-fraction (572 g/kg) and lower C-fraction (45.3 g/kg) in WPS silage (R7 phenological stage) either in control group or treated with microbial inoculants. Contrasting with the current study, de Morais et al. [7] observed a decrease in C-fraction proportion when microbial inoculants were incorporated into the silage stored for 120 d, which was associated with reduced fermentation losses and dilution of low degradable protein fraction. Differences in protein fractions between studies may be related to soybean cultivar, phenological stage at which plants were harvested, and crop conditions. In the current study, the lowest DM losses were observed in silages treated with chemical additives, which supports the decrease in silage C-fraction proportion with FA-type and PA-type additives.

Additives, especially the chemical additives, increased A-fraction and thereby decreased B-fraction proportion (dilution effect) between 120 and 150 d of silage storage length. Thereby, in general, chemical additives increase protein-effective degradation. Controversially, Junges et al. [42] reported that less than 5% of silage proteolysis is directly associated with acid production, and lower proteolysis has been well documented in lower-pH silages [43,44]. We are unaware of studies that evaluated the effects of chemical

additives with organic acids on WPS silage protein fractions and solubility. Besides reduced NH3-N observed in acid-treated silos, structural changes induced by acid addition reduced estimates of effective degradation in the current study.

5. Conclusions

Chemical additives were effective in reducing silage pH and NH_3-N concentration at silo openings, suggesting that fermentation of ensiled material stabilized earlier than other treatments and resulted in lower DM losses, greater protein concentrations, and reduced undigestible proportions of protein WPS silage. All silage additives were able to increase lactate and NFC concentrations.

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