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RESEARCH ARTICLE

Effect of *Lactobacillus buchneri* and sodium benzoate on the fermentative profile, bacterial taxonomic diversity, and aerobic stability of sorghum silages at different fermentation times

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ABSTRACT

The combined use of a microbial additive and a chemical additive in sorghum (*Sorghum bicolor* (L.) Moench) silages would reduce fermentation losses, favoring aerobic stability by modulating a favorable microbiome to control those losses. The purpose of this study was to evaluate the action of *Lactobacillus buchneri* and sodium benzoate on the fermentative profile, bacterial taxonomic diversity and aerobic stability of sorghum silages at different fermentation times. A completely randomized experimental design was adopted in a 4 × 4 factorial scheme, in order to evaluate four additives in sorghum silage (Control treatment, *L. buchneri*, sodium benzoate and *L. buchneri* combined with sodium benzoate) and four opening times (30, 60, 90 and 120 d fermentation), in five replicates. Silages inoculated with sodium benzoate showed higher pH values (3.70) at 90 d of fermentation, not differing (P > 0.05) from control (CTR) silage and the other silages. The largest lactic acid bacteria populations were observed at 120 d of fermentation in the silages inoculated with *L. buchneri* (9.06) followed by the silages inoculated with sodium benzoate and with the combination *L. buchneri* + sodium benzoate (8.37 and 8.85 log CFU, respectively) at 90 d of fermentation. For gas losses, in which silage treated with sodium benzoate showed lower values, with an average of 0.72% DM. The silages presenting a high DMR standard, ranging from 94.6% to 98.0%, with the exception of the CTR silages. Silages treated with *L. buchneri* showed higher values (98%) for the occurrence of the genus *Lactobacillus* in both fermentation times. The combined action of *L. buchneri* and sodium benzoate kept the *Lactobacillus* population stable at 30 and 120 d of fermentation. The combination of *L. buchneri* and sodium benzoate improved the fermentative profile and chemical composition of sorghum silages, reducing losses and increasing aerobic stability after 120 d of fermentation.

Key words: Chemical additive, heterofermentative inoculant, microbial population, 16S ribosomal rRNA, *Sorghum bicolor*.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) has been widely used for silage making in areas of irregular rainfall distribution. When compared to other crops, such as corn, sorghum is more efficient in water use and tolerates low soil moisture conditions (McCary et al., 2020), besides presenting higher DM yields per hectare under adverse conditions, characterizing it as an important forage resource for regions of low rainfall occurrence (Fernandes et al., 2022).

Usually, when at the ideal point for harvest, the forage sorghum presents very high levels of soluble carbohydrates, showing values above 15% DM (Lucena et al., 2021), which can compromise the fermentation of silages, leading to pH values below those recommended, and allowing the activity of secondary microorganisms such as yeasts (Santos et al., 2021), that can rapidly multiply and produce ethanol, causing greater losses in these silages and deterioration when exposed to air, due to the assimilation of lactate and residual soluble carbohydrates (Kung et al., 2018). Kung et al. (2018) reported that heterofermentative bacteria can inhibit the activity of yeasts that initiate the deterioration process, from the production of acetic acid that has antifungal properties.

However, information on this phenomenon is still controversial in the literature, since the inoculation of *Lactobacillus buchneri* alone may not be able to control losses, often having its positive result restricted to maintain aerobic stability. In their study, Santos et al. (2021) reported positive effects on forage sorghum silages when they combined urea and *L. buchneri*, which resulted in high DM recovery rates, aerobically stable silages, and even better animal performance in comparison to the use of *L. buchneri* alone.

Considering the information above, it was hypothesized in this research that the combined use of a microbial additive and a chemical additive in sorghum silages would reduce fermentation losses, favoring aerobic stability by modulating a favorable microbiome to control those losses.

Sodium benzoate has been identified as a highly efficient chemical additive in controlling losses and increasing aerobic stability in silages by reducing the yeast population, and it is also associated with reductions in proteolysis during fermentation (Silva et al., 2015). However, the effect of this additive combined with *L. buchneri* on forage sorghum silages is not yet known, which in a way, is very important in farms that produce silages on a large scale.

Therefore, the purpose of this study was to evaluate the effect of *Lactobacillus buchneri* and sodium benzoate on the fermentation profile, bacterial taxonomic diversity, and aerobic stability of sorghum silages in different fermentation times.

MATERIALS AND METHODS

Local considerations for experiment execution

The experiment was carried out at the Federal University of Piauí, in the city of Bom Jesus (09°04′28″ S, 44°21′31″ W; 277 m a.s.l.), with climate classified as AW with a dry winter season; and in the Forage Crops Laboratory of the Agricultural Sciences Center of the Federal University of Paraíba (6°58′07″ S, 35°44′03″ W; 627 m a.s.l.)

A hybrid of forage sorghum (S520) (*Sorghum bicolor* (L.) Moench) developed by Embrapa Corn and Sorghum was planted in an area of 400 m², after soil analysis and correction through phosphate fertilization with single superphosphate during planting and N fertilization with urea 15 d after plant germination. The sowing was carried out with 0.7 m spacing between rows, using 12 pure and viable seeds per linear meter, during November 2019.

During the period of plant development, the area was daily irrigated with a fixed sprinkler system equivalent to 5 mm d^{-1} until the day of harvest, when it was harvested at the grain stage of physiological maturity, with an approximate DM content of 30%, respecting the ideal state of maturation for ensiling the plant, which occurs around 100 d after planting.

Experimental design and ensiling process

A completely randomized design was adopted, in a 4 × 4 factorial scheme with four treatments and five replicates, evaluating four additives in the sorghum silage: Control treatment (CTR), *Lactobacillus buchneri*, sodium benzoate, and *L. buchneri* combined with sodium benzoate; and four opening times (30, 60, 90 and 120 d of fermentation). The sorghum was ensiled in experimental 7.5 L silos, equipped with Bunsen valve in the lid for gas escape, adopting a particle size of 2 cm and an approximate density of 600 kg m^{-3}. The application of the chemical and microbiological additives was carried out in the sorghum forage mass according to the treatment. The inoculation occurred according to the manufacturer's specifications. In addition, a volume of 120 mL of distilled water was used in the ensiled mass of the CTR treatment in order to isolate the effect of water. *L. buchneri* was applied following the manufacturer's recommendations (Lallemand Animal Nutrition Brasil, Aparecida de Goiânia, Goiânia, Brazil), at a concentration of 105 CFU g^{-1} fresh mass, while sodium benzoate was applied at 1 g kg^{-1} on a fed basis.

Silage quality parameters

The pH values were determined by collecting approximately 25 g sample of the ensiled material from each treatment and adding 100 mL water. After 1 h, the reading was taken using a potentiometer according to the methodology of Bolsen et al. (1992). It was followed for the determination of N-NH_3 in the samples for methodology of Bolsen et al. (1992). For the analysis of organic acids, lactic acid (LA), acetic acid (AA), and propionic acid (PA), it was used a high performance liquid chromatograph (HPLC) (Shimadzu, Dallas, Texas, USA), model SPD-10^a VP coupled to an ultra violet detector (UV) using a wavelength of 210 nm; C18 reversed phase column (Supelco, Bellefonte, Pennsylvania, USA); 30 cm × 4.5 mm in diameter; column flow 0.6 mL min^{-1}; column pressure 87 kgf; mobile phase water in 1% sulfuric acid and injected volume 10 µL. Ten grams of sample were removed and diluted in 90 mL distilled water and filtered through Whatman filter paper (Kung and Ranjit, 2001).

The buffer capacity was determined according to Playne and McDonald (1966), where 20 g fresh sample were weighed, macerated with 250 mL distilled water, placed in pots at room temperature until reading, and determined by a potentiometer. The soluble carbohydrates content was determined through the concentrated sulfuric acid method, as described by Dubois et al. (1956). Samples were collected at the moment of ensiling to determine the chemical composition of the sorghum (Table 1). The DM losses in the silages through gas and effluent were quantified by weight difference as described by Jobim et al. (2007).

Table 1. Chemical composition of sorghum silages inoculated with chemical (sodium benzoate) and microbial (*Lactobacillus buchneri*) additives during ensiling. CTR: Control treatment.

	Treatments			
Variables (g kg^{-1} DM)	CTR	*L. buchneri*	Sodium benzoate	*L. buchneri* + sodium benzoate
Dry matter	281.20	273.30	293.20	307.70
Crude protein	43.80	40.02	41.40	34.70
Ethereal extract	33.80	25.00	24.90	21.90
Neutral detergent fiber	590.07	571.90	572.50	570.12
Acid detergent fiber	310.03	324.40	297.70	324.43
Organic matter	936.30	925.80	947.60	940.08
Mineral matter	637.00	742.00	524.40	592.02
Buffer capacity	8.18	10.04	12.70	13.24
Water soluble carbohydrates	120.17	130.12	160.00	160.43

Microbial populations

A 25 g fresh sample was collected and 225 mL sterile phosphate buffer solution were added. After processing on a type homogenizer Stomacher (maximum pressure up to 207 MPa, BIOBASE GROUP Jinan, Shandong, China) for 1 min, 1 mL mixture was pipetted, and serial dilutions were performed to count lactic acid bacteria (10^{-3} to 10^{-7}), and molds and yeasts (10^{-2} to 10^{-6}).

For lactic acid bacteria (LAB) counting, samples were plated in MRS broth (Difco, Detroit, Michigan, USA) using Petri dishes and incubated in biochemical oxygen demand (BOD) test at 37 °C for 48 h before counting. Molds and yeasts (MY) were quantified using the pour-plating technique on potato dextrose agar (Difco) which was acidified by 1.5% of 10% tartaric acid (w/v) and plates were incubated in BOD at 25 °C for 5 d. Plates were considered susceptible to counting when there were values between 30 and 300 CFU (colony forming units) in the Petri dish.

Aerobic stability

The aerobic stability was determined in 2 kg silage samples taken when silos were opened at 90 and 120 d after ensiling and relocated to clean silos without compaction. A thermometer was attached to the geometric center of the mass of each silo and the temperature was recorded every 30 min using a digital thermometer (Incoterm, China. Temperature range -50 to 300 ºC). Room temperature was recorded on a thermometer and was controlled with the aid of an air conditioner at 25 °C. The aerobic stability was calculated as the number of hours for the ensiled mass to remain up to 2 °C in relation the room temperature (Kung et al., 2000).

Silage's bacterial community analysis through metataxonomics of 16S ribosomal rRNA gene using high-performance sequencing

Bacterial community analyses were performed at two fermentation times in all treatments, 30 and 120 d, to observe the stabilization of the microbial ecology over time. At the moment of ensiling, only CTR silages were collected to represent the treatments before fermentation, since the in natura material had not had effect yet.

The DNA extraction from silage samples was carried out using a commercial kit (Power Soil DNA Isolation kit, MoBio, Carlsbad, California, USA), according to the manufacturer's recommendations. The V3-V4 region of the 16S RNAr was amplified through PCR (95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min) using the 16S Amplicon PCR Forward Primer = 3' and 16S Amplicon PCR Reverse Primer = 5'. The PCR was performed in triplicate, in a final volume of 25µL containing 12.5 µL 2x kapa hifi hotstart readmix, 5 µL each primer and 2.5 ng template DNA. The purified PCR products were quantified through fluorometry using Qubit 3.0 (Life Invitrogen, Waltham, Massachusetts, USA).

The library was prepared using the adapters of the Nextera XT sample Prep Kit (Illumina, San Diego, California, USA). Subsequently, the DNA fragments were purified with Agencourt AMPure XP reagent (Beckman Coulter, Indianapolis, Indiana, USA). After purification, the library was validated in the Fragment Analyzer (Agilent Technologies, Santa Clara, California, USA).

The number of sequences per sample was normalized to 14 900 reads to perform the analyses of ecological diversity alpha (richness uniformity) and beta (principal component analysis, PCA); aligned by the MAFFT (Katoh et al., 2002) and then used for the construction of the phylogenetic tree by FastTree2 (Price et al., 2010). The visualizations of the taxonomic composition, especially relative abundance and alpha diversity were made using the phyloseq package v. 1.8.2 (McMurdie and Holmes, 2013) of the RStudio software v.3.5.7 (Posit PBC, Chicago, Illinois, USA). The taxonomic classification was attributed using the Naïve Bayes method on the trained database of SILVA v. 132 with 99% for region V3-V4 (Quast et al., 2013). The assessment of alpha diversity was made by estimating the ecological indexes of richness and uniformity of the communities, respectively, Chao1 and Simpson.

Chemical composition

The chemical composition analysis was performed according to AOAC (1990), the samples were subjected to pre-drying in a forced ventilation oven at 55 ºC for 72 h. Soon after, they were processed in a mill with sieve of 1 mm and were used to determine DM contents; mineral matter (MM), and ether extract (EE) were determined according to AOAC (1990); while the crude protein (CP) content in DM was determined through the micro Kjeldahl method, according to AOAC (1990); and the concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF), were determined according to the techniques described by Van Soest et al. (1991).

Statistical analysis

The data were subjected to ANOVA, and when significant differences were detected, the means were compared through the Tukey's test at the 0.05 probability level. For the variables which the interaction effect was significant, it was decomposed the effect of each silage within each opening time. Using the SAS System for Windows 9.4 (SAS Institute, Cary, North Carolina, USA).

The alpha diversity indexes were assessed using the paired Kruskall-Wallis test, while the dissimilarity between treatments was assessed by the multivariate permutation method (PERMANOVA). Hierarchical cluster and random forest heatmap analyses were performed using an R-based statistical tool.

RESULTS

It was found interaction effect ($P < 0.05$) on pH values, populations of lactic acid bacteria (LAB), yeasts, ammonia N ($N\text{-}NH_3$) and aerobic stability. Silages inoculated with benzoate showed higher pH values (3.70) at 90 d of fermentation, not differing ($P > 0.05$) from CTR silage and the other silages (Table 2). The largest LAB populations were observed at 120 d of fermentation in the silages inoculated with *L. buchneri* (9.06) followed by the silages inoculated with sodium benzoate and with the combination *L. buchneri* + sodium benzoate (8.37 and 8.85 log CFU, respectively) at 90 d of fermentation. Regarding the yeasts count, the lowest populations were observed at 30 d, remaining stable until 60 d in the silages inoculated with *L. buchneri* (1.87 and 1.67 CFU) respectively.

It was found effect of interaction ($P < 0.05$) on the contents of lactic, acetic, propionic and butyric acids (Table 3). Regarding the contents of lactic acid, silages treated with the combination *L. buchneri* + sodium benzoate showed higher values above 60 d of fermentation (99.57 g kg^{-1}), not differing from the subsequent opening times. The silages treated with sodium benzoate showed the highest lactic acid values at 60 d of fermentation (95.24 g kg^{-1}), with a slight decrease in these levels as the fermentation time passed.

The acetic acid levels were higher in silages treated with *L. buchneri* after 60 d (36.16 g kg^{-1} DM), followed by silages treated with sodium benzoate and with the combination *L. buchneri* + sodium benzoate, which were similar to each other (27.03 and 27.82 g kg^{-1} DM, respectively), in addition, the levels of propionic acid showed the same behavior. Lower values of butyric acid were observed in the silages treated with the combination *L. buchneri* + sodium benzoate (1.53 g kg^{-1} DM) followed by silages treated with *L. buchneri* and with sodium benzoate (2.20 and 2.05 g kg^{-1} DM, respectively).

There was a difference ($P = 0.01$) for gas losses, in which silage treated with benzoate showed lower values, with an average of 0.72% DM (Table 4). The silages presenting a high DMR standard, ranging from 94.60% to 98.00%, with the exception of the CTR silages. The additives used, improved aerobic stability over the days of fermentation. Differences were observed ($P < 0.05$) on the contents of DM, crude protein, ether extract, neutral detergent fiber, buffer capacity and soluble carbohydrates. The silages treated with the combination *L. buchneri* + sodium benzoate showed a mean DM value of 324 g kg^{-1}, which was superior to the silages treated with *L. buchneri* and with sodium benzoate separately (287.0 and 303.0 g kg^{-1}, respectively). The crude protein, higher values were observed in silages treated with *L. buchneri*, sodium benzoate and with their combination (39.90, 40.10, 40.04 g kg^{-1}, respectively).

Table 2. pH Values, microbial populations and N-NH_3 content in sorghum silages treated with chemical (sodium benzoate) and microbial (*Lactobacillus buchneri*) additives at different fermentation times. Different lowercase letters in the row represent significance of $P < 0.05$ for use of additive; different uppercase letters in the column represent significance of $P < 0.05$ for silo opening. Addit × Time: Interaction effect between additive and fermentation time; CTR: control treatment; SEM: standard error of the mean.

Fermentation time (d)	Treatments				Average
	CTR	*L. buchneri*	Sodium benzoate	*L. buchneri* + Sodium benzoate	
			pH		
30	3.60^{baA}	3.50^{bA}	3.62^{abAB}	3.65^{aA}	3.56
60	3.45^{aB}	3.30^{bB}	3.42^{abC}	3.40^{abB}	3.48
90	3.65^{aA}	3.50^{bA}	3.70^{aA}	3.60^{abA}	3.56
120	3.55^{abAB}	3.62^{aA}	3.52^{abBC}	3.45^{bB}	3.52
Average	3.59	3.39	3.61	3.53	
			Lactic acid bacteria (log CFU g^{-1})		
30	6.84^{aA}	6.69^{aBC}	6.54^{aB}	7.74^{aAB}	6.23
60	4.24^{cB}	5.58^{bcC}	7.22^{aAB}	6.48^{abB}	7.15
90	7.40^{abA}	7.28^{bB}	8.37^{abA}	8.85^{aA}	7.44
120	6.44^{bA}	9.06^{aA}	7.64^{abAB}	7.34^{bAB}	7.54
Average	6.88	7.16	7.97	7.62	
			Yeasts (log CFU g^{-1})		
30	6.56^{aA}	1.87^{bA}	4.88^{aA}	2.09^{bB}	5.80
60	5.00^{aA}	1.67^{bA}	5.39^{aA}	4.80^{aAB}	2.22
90	6.81^{aA}	2.37^{bA}	5.51^{aA}	5.74^{aA}	5.11
120	4.82^{aA}	2.96^{aA}	4.64^{aA}	4.43^{aAB}	4.26
Average	5.82	2.90	5.11	4.21	
			Molds (log CFU g^{-1})		
30	1.00	1.40	1.84	1.62	1.46^{B}
60	1.14	2.24	3.46	4.60	2.86^{AB}
90	1.14	2.24	3.46	4.60	2.85^{AB}
120	4.25	2.18	4.55	3.18	3.79^{A}
Average	1.88^{c}	2.00^{b}	3.33^{a}	3.50^{a}	
			N-NH_3 (% N total)		
30	0.67^{abA}	0.45^{cB}	0.50^{bcB}	0.75^{aA}	0.67
60	0.72^{abA}	0.77^{aA}	0.70^{abA}	0.57^{bAB}	0.58
90	0.64^{aA}	0.60^{aAB}	0.57^{aAB}	0.50^{aB}	0.51
120	0.65^{aA}	0.52^{aB}	0.57^{aAB}	0.50^{aB}	0.57
Average	0.58	0.069	0.058	0.056	

Variables	P-value			SEM
	Additive	Time	Addit × Time	
pH	< 0.01	< 0.01	< 0.01	0.01
Lactic acid bacteria	< 0.01	< 0.01	< 0.01	0.20
Yeasts	< 0.01	0.10	0.04	0.36
Molds	0.02	< 0.01	0.33	0.43
N-NH_3	0.02	< 0.01	< 0.01	0.02

Table 3. Organic acid contents in sorghum silages treated with chemical (sodium benzoate) and microbial (*Lactobacillus buchneri*) additives at different fermentation times. Different lowercase letters in the row represent significance of P < 0.05 for use of additive; different uppercase letters in the column represent significance of P < 0.05 for silo opening. CTR: Control treatment; SEM: standard error of the mean.

Fermentation time (d)	Treatments				Mean
	CTR	*L. buchneri*	Sodium benzoate	*L. buchneri* + Sodium benzoate	
			Lactic acid (g kg^{-1})		
30	53.09^{Bb}	57.50^{Bb}	59.93^{Ab}	73.78^{Ab}	61.07
60	55.84^{Ba}	65.01^{Ba}	95.24^{Aa}	99.57^{Aa}	78.91
90	65.94^{Ba}	62.28^{Ba}	59.07^{Bb}	97.42^{Aa}	71.17
120	56.62^{Ba}	56.06^{Bb}	62.89^{Bb}	87.40^{Aa}	65.74
Mean	57.87	60.21	69.28	89.54	
			Acetic acid (g kg^{-1})		
30	18.10^{Bb}	26.89^{Ac}	20.48^{Bc}	21.57^{Bc}	21.76
60	17.49^{Cb}	36.16^{Ab}	25.95^{Bb}	29.25^{Bb}	27.21
90	24.43^{Ca}	40.67^{Aa}	32.67^{Ba}	32.03^{Ba}	32.45
120	20.11^{Bb}	30.91^{Ab}	29.04^{Aa}	28.44^{Ab}	27.12
Mean	20.03	33.65	27.03	27.82	
			Propionic acid (g kg^{-1})		
30	3.00^{Bb}	8.78^{Ab}	4.62^{Bc}	2.43^{Bc}	5.99
60	4.72^{Cb}	10.08^{Aa}	7.45^{Bb}	7.37^{Bb}	7.40
90	7.01^{Ba}	11.27^{Aa}	9.60^{Aa}	11.25^{Aa}	9.78
120	6.21^{Ba}	9.67^{Ab}	10.27^{Aa}	10.46^{Aa}	9.15
Mean	5.23	9.78	7.98	7.87	
			Butyric acid (g kg^{-1})		
30	1.71^{Ab}	1.91^{Aa}	1.09^{Aa}	0.88^{Ab}	1.39
60	2.61^{Ab}	2.35^{Aa}	1.95^{Aa}	1.43^{Aa}	1.83
90	3.34^{Ab}	2.41^{Aa}	2.25^{Aa}	1.65^{Aa}	2.16
120	4.12^{Aa}	2.14^{Ba}	2.94^{Ba}	2.16^{Ba}	2.84
Mean	2.94	2.20	2.05	1.53	

Variables	P-value			SEM
	Additive	Time	Addit × Time	
Lactic acid	< 0.01	< 0.01	< 0.01	1.96
Acetic acid	< 0.01	< 0.01	< 0.01	0.49
Propionic acid	< 0.011	< 0.01	< 0.01	0.31
Butyric acid	0.649	< 0.01	< 0.01	0.12

Table 4. Fermentation losses in sorghum silages treated with chemical (sodium benzoate) and microbial (*Lactobacillus buchneri*) additives at 120 d fermentation. Different lowercase letters in the row represent significance of P < 0.05). CTR: Control treatment; SEM: standard error of the mean; AF: as fed basis (AF).

Variables	Treatments				Mean	SEM	P-value
	CTR	*L. buchneri*	Sodium benzoate	*L. buchneri* + Sodium benzoate			
Gas losses, % DM	1.03^{ab}	1.18^{a}	0.72^{b}	1.07^{ab}	1.00	0.07	< 0.01
Effluent losses, kg AF t^{-1}	56.70	47.70	45.50	45.60	48.90	5.72	0.49
Total losses, %	5.95	5.71	5.27	5.63	5.64	0.26	0.40
Dry matter recovery, %	87.30	94.30	94.60	98.00	93.50	3.22	0.19

The CTR silage showed a more diverse microbiota during ensiling and fermentation times. In addition, the silages treated with *L. buchneri* showed dissimilarity at 30 d of fermentation when compared to 120 d (Figures 1 and 2). The taxonomic depth of the bacterial groups was characterized by the dominance of four phyla: *Actinobacteriota*, *Bacteroidota*, *Firmicutes*, and *Proteobacteria* (Figure 3). As for genus, nine types of genera were identified, from which the most abundant were *Acetobacter*, *Lactobacillus*, and *Lactococcus*. The silages treated with *L. buchneri* showed higher values (98%) for the occurrence of the genus *Lactobacillus* in both fermentation times (Figure 4). The machine learning method classification of the type Random Forest, showed that at 120 d of opening, populations of strains 3260 and 3084 of *L. buchneri* dominated the environment in silages treated with *L. buchneri*, sodium benzoate, and their combination, showing frequencies ranging from 2.5 to 3.5 $\log^{10}$ (Figure 5).

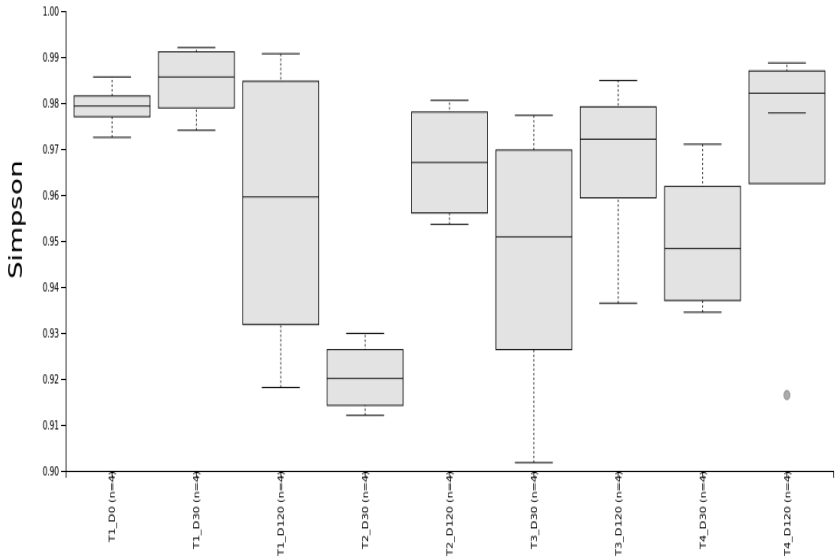

Figure 1. Diversity index (Simpson) of sorghum silages with chemical and microbial additives at 0, 30 and 120 d fermentation. T1 D0: control treatment (ensiling); T1 D30: control treatment (30 d fermentation); T1 D30: control treatment (120 d fermentation); T2 D30: *Lactobacillus buchneri* (30 d fermentation); T2 D120: *L. buchneri* (120 d fermentation); T3 D30: Sodium benzoate (30 d fermentation); T3 D120: Sodium benzoate (120 d fermentation); T4 D30: *L. buchneri* + Sodium benzoate (30 d fermentation); T4 D120: *L. buchneri* + Sodium benzoate (120 d fermentation).

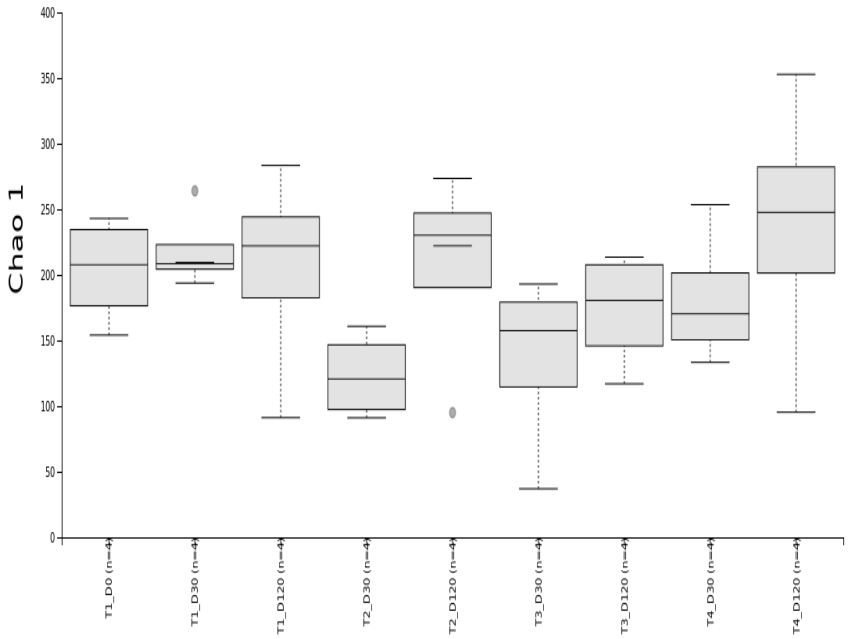

Figure 2. Richness index (Chao 1) of sorghum silages with chemical and microbial additives at 0, 30 and 120 d fermentation. T1 D0: Control treatment (ensiling); T1 D30: control treatment (30 d fermentation); T1 D30: control treatment (120 d fermentation); T2 D30: *Lactobacillus buchneri* (30 d fermentation); T2 D120: *L. buchneri* (120 d fermentation); T3 D30: Sodium benzoate (30 d fermentation); T3 D120: Sodium benzoate (120 d fermentation); T4 D30: *L. buchneri* + Sodium benzoate (30 d fermentation); T4 D120: *L. buchneri* + Sodium benzoate (120 d fermentation).

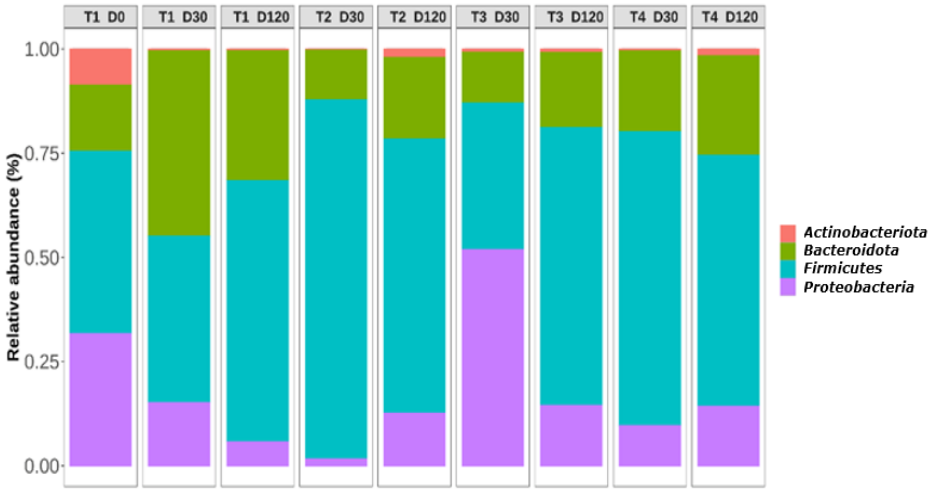

Figure 3. Relative abundance of the main phyla in sorghum silage with chemical and microbial additives at 0, 30 and 120 d fermentation. T1 D0: Control treatment (ensiling); T1 D30: control treatment (30 d fermentation); T1 D30: control treatment (120 d fermentation); T2 D30: *Lactobacillus buchneri* (30 d fermentation); T2 D120: *L. buchneri* (120 d fermentation); T3 D30: Sodium benzoate (30 d fermentation); T3 D120: Sodium benzoate (120 d fermentation); T4 D30: *L. buchneri* + Sodium benzoate (30 d fermentation); T4 D120: *L. buchneri* + Sodium benzoate (120 d fermentation).

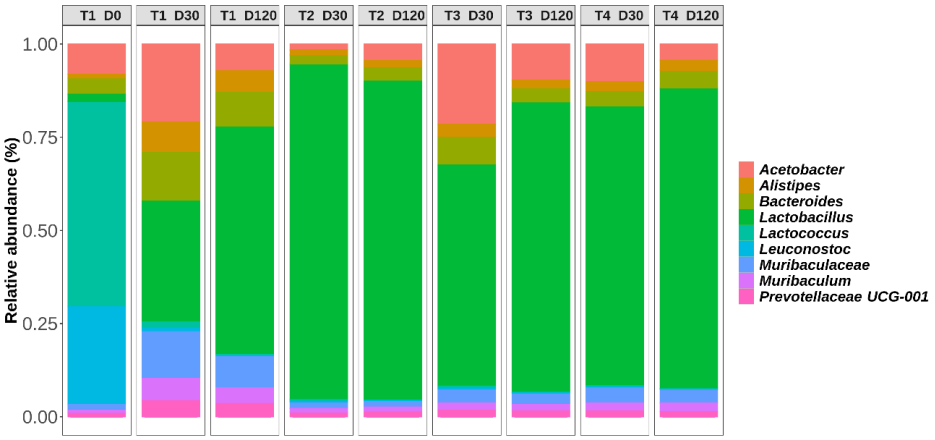

Figure 4. Relative abundance of the main genera in sorghum silage with chemical and microbial additives at 0, 30 and 120 d fermentation. T1 D0: Control treatment (ensiling); T1 D30: control treatment (30 d fermentation); T1 D30: control treatment (120 d fermentation); T2 D30: *Lactobacillus buchneri* (30 d fermentation); T2 D120: *L. buchneri* (120 d fermentation); T3 D30: Sodium benzoate (30 d fermentation); T3 D120: Sodium benzoate (120 d fermentation); T4 D30: *L. buchneri* + Sodium benzoate (30 d fermentation); T4 D120: *L. buchneri* + Sodium benzoate (120 d fermentation).

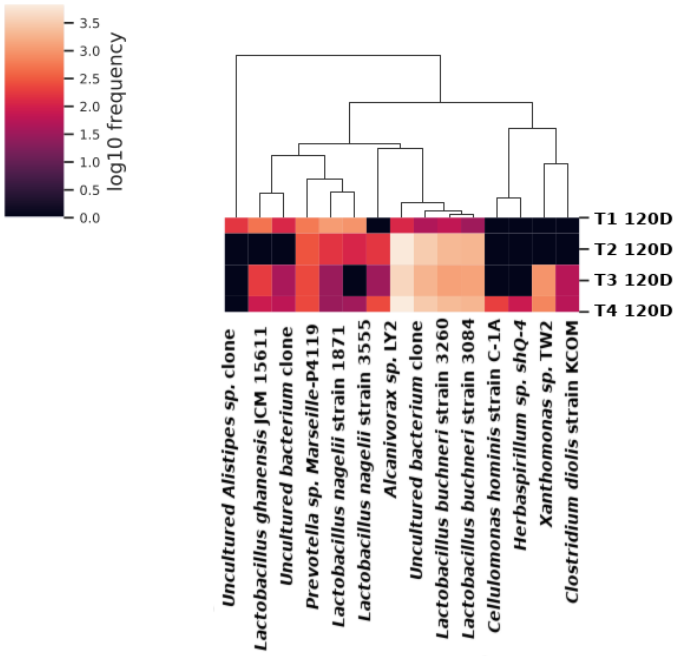

Figure 5. Random Forest-type heat map of bacterial communities to sorghum bacterial community with chemical and microbial additives at 0, 30, and 120 d fermentation. T1 D120: Control treatment (120 d fermentation); T2 D120: *Lactobacillus buchneri* (120 d fermentation); T3 D120: Sodium benzoate (120 d fermentation); T4 D120: *L. buchneri* + Sodium benzoate (120 d fermentation).

DISCUSSION

All silages presented pH values below the ideal range recommended by Kung et al. (2018), which is from 3.8 to 4.2. In fact, forage sorghum silages are known for their high soluble carbohydrate content and low buffering power, which culminates in a fast pH drop in the early stages of fermentation (Santos et al., 2021). The silages treated with additives increased the population of LAB even when ensiled for a long period (120 d), which can be explained by the presence of *L. buchneri* that provided a suitable fermentative environment to enhance the growth of these microorganisms. The opposite effect occurred for cultivable mold and yeast populations, that is, these populations caused little damage to the ensiling process, being controlled also by *L. buchneri* at 120 d of fermentation and by sodium benzoate, both separately. Also, despite the higher carbohydrate content, the additives were able to control the yeast population and improved the aerobic stability.

This effect occurred because silages treated with the inoculants, especially *L. buchneri*, increase acetic acid concentrations, which may have reduced the activity of fungi and yeasts, but it is clear that the combination of a chemical inoculant with a microbiological inoculant was decisive in inhibiting these microorganisms in a shorter period, since *L. buchneri* contributes to the production of acetic acid, whereas the sodium benzoate dissociates during ensiling releasing benzoic acid, which has inhibitory action on yeasts and fungi (Santos et al., 2019).

Thus, the silages inoculated with *L. buchneri* presented the lowest concentration of lactic acid and the highest concentrations of acetic and propionic acid among the treated silages. This reduction is explained by the fact that heterofermentative bacteria of the species *L. buchneri* use this acid for their growth, thus producing acetic acid as metabolic waste, and 1,2-propanediol, propionic acid and carbon dioxide in smaller proportions.

It was noted that the combined action of *L. buchneri* and sodium benzoate was able to reduce these values at 90 d of fermentation, which is important for sorghum silage production at the farm level, since the literature reports that the fermentation time can increase ammonia content due to protein solubilization during the fermentation process for sorghum silages. This combined action also resulted in high DM recovery values (98%) and low losses.

As expected, sodium benzoate reduced the rate of losses through gas in treated silages, due to possible inhibition of proteolysis. The combination *L. buchneri* + sodium benzoate caused the best results for the fermentation pattern and aerobic stability, probably as a direct effect of suppressing yeast growth in the silage, besides maintaining the nutritional value of the silages. In the present study, the aerobic stability only reached 144 h (Table 5) when *L. buchneri* and sodium benzoate were added in the silage, which remained over the time of air exposure, indicating that this combination has associative effects that intensify the retardation of the reactions that heat the ensiled mass, and that the effect of the inoculant can be potentiated by antifungal chemical agents.

Table 5. Aerobic stability of sorghum silages treated with chemical (sodium benzoate) and microbial (*Lactobacillus buchneri*) additives at 90 and 120 d fermentation. Different lowercase letters in the row represent significance of $P < 0.05$ for use of additive; different uppercase letters in the column represent significance of $P < 0.05$ for silo opening. CTR: Control treatment; SEM: standard error of the mean.

Fermentation time	Treatments				Mean
	CTR	*L. buchneri*	Sodium benzoate	*L. buchneri* + Sodium benzoate	
d	Aerobic stability (h)				
90	12.00^{cB}	27.80^{bB}	33.40^{bB}	44.20^{aB}	29.35
120	31.40^{cA}	72.40^{bA}	88.90^{bA}	144.08^{aA}	84.37
Mean	21.7	50.1	61.1	94.5	
	P-value				
	Additive		Time	Addit × Time	SEM
Aerobic stability	< 0.01		< 0.01	< 0.03	5.99

The DM contents found in the treated silages, remained within the range considered ideal, which is between 28% and 32% (Kung et al., 2018), showing that the additives fulfilled their role of maintaining these values. The CTR silage showed a reduction of one percentage point below the plant material. Thus, these variations observed between in natura, and post-fermentation sorghum can be attributed to losses during the fermentation process. Therefore, that reduction may be due to the consumption of soluble carbohydrates during fermentation, explaining the lower DM values. In their study, Junges et al. (2017) reported that under high moisture conditions, plant and bacterial enzymes mediate approximately 90% of proteolysis in silages.

Similarly, Fernandes et al. (2022) also reported that the moisture and storage time in silages not treated with additives, proved to be the most important factors for protein losses, because these losses suggest activity of enterobacteria and in some cases of clostridia. Therefore, the studied additives showed to be efficient in maintaining the levels of protein, with no differences among themselves. The concentrations of NDF had a slight reduction in the silages treated with sodium benzoate and with the combination of additives, as well as high recovery rates of soluble carbohydrates, possibly due to the effect of acid and enzymatic hydrolysis of hemicellulose.

The alpha diversity was higher at the moment of ensiling, as there was a high diversity of epiphytic microorganisms in the sorghum, which was already expected since the environment at that moment was still aerobic and still had a high pH. However, after ensiling, Simpson and Chao 1 indexes were lower in the silages treated with additives, which is explained by the fact that fermentation becomes intense when oxygen is fully expelled from the silo, so the complex microbial communities are gradually replaced by LAB, an effect that was potentiated by the inoculation with *L. buchneri*, along with considerable reductions in microbial diversity by the antimicrobial action of the chemical additive, which is a requirement for a successful ensiling process (Kung et al., 2018).

The addition of *L. buchneri* and sodium benzoate in the sorghum silages, may have inhibited the growth of other microorganisms, resulting in reduced species diversity. The reactions of the tested additives, produced substances that contributed to the suppression of undesirable microorganisms, as evidenced by low losses through proteolysis, high recoveries of residual carbohydrates and high aerobic stability, which explains the appearance of bacteria of the genus *Acetobacter* that takes advantage of carbohydrates to produce acetic acid. Duae et al. (2021) cited that when LAB strains are exogenous in the ensiling process, they proliferate rapidly and become the dominant species in bacterial communities.

The diversity, function, and structure of microbial communities are important topics in microbial ecology and have been the focus of silages research. Based on the literature, this is the first research reporting the microbial community of forage sorghum silages treated with a combination of *L. buchneri* and sodium benzoate, evaluated at different fermentation times. The Proteobacteria dominated the microbiome at the beginning of ensiling, while Firmicutes dominated the silages.

Such changes in reduced richness and uniformity are expected because most of the populations that compose the phylum Firmicutes are native bacteria of the plant itself or the soil, which are mainly obligate aerobic microorganisms that decrease when the silo is closed (Da Silva et al., 2021). The same authors also observed this change in bacterial composition over the fermentation days, when they evaluated corn silages inoculated with *L. buchneri* and *L. hilgardii*.

As fermentation progressed, all silages showed a microbiome dominated by Firmicutes. Also, *L. buchneri* increased these populations starting at 30 d of fermentation and decreasing these populations at 120 d. On the other hand, the action of sodium benzoate required more time for the dominance of the genus *Lactobacillus*. However, when the combination of these two additives was tested, it was possible to observe dominant populations at 30 d, persisting until 120 d of fermentation.

The literature reports that in cases like this, the combination of chemical and microbial additives promotes a correction in *Lactobacillus* populations, by increasing them to maximum in relatively short periods, and extending to longer fermentation times. In addition, a possible mechanism by which *Lactobacillus* can modulate the bacterial population during the fermentation process, along with the

reduction of pH, may be the high ability of this group to release compounds of antagonistic action, which can even inhibit populations of halotolerant bacteria.

This partly explains the absence of genus *Lactococcus* in the inoculated silages but dominated the CTR silage due to the higher epiphytic abundance in the sorghum plant. In general, *Lactococcus* are major producers of lactic acid, initiating fermentation during the early stages of ensiling, and they are also responsible for fermentation progression and pH reduction, growing rapidly to reach high abundance.

Liu et al. (2019) cited that this early phenomenon in the fermentative process is important for the succession of the genus *Lactobacillus* in general, as it may be a precursor for its dominance. Interestingly, there are considerable differences between the dominant bacterial communities when comparing different studies that have accessed the microbiome of bacteria in silage, mainly depending on the type of forage ensiled, but in most cases, genera *Lactobacillus*, *Pediococcus* and *Lactococcus* have usually been the predominant ones (Da Silva et al., 2021; Duae et al., 2021).

The strains identified in the present study are classified by National Center for Biotechnology Information (NCBI) as bacteria that act by restricting the action of yeasts and clostridia. Therefore, these findings support the hypothesis that the combination of *L. buchneri* and sodium benzoate produces enough conditions to block the action of organisms that impair fermentation. The occurrence of the succession from a microbiome dominated by Proteobacteria to a microbiome dominated by Firmicutes, mainly by genus *Lactobacillus*, was fundamental for the course of fermentation in the sorghum silages, reducing losses and improving aerobic stability when treated with the combination *L. buchneri* + sodium benzoate.

CONCLUSIONS

The combination of *Lactobacillus buchneri* and sodium benzoate improves the fermentative profile and chemical composition of sorghum silages, reducing losses and increasing aerobic stability at 120 d, favoring a bacterial community dominated by *Lactobacillus*.

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Author contribution

Conceptualization: E.M-S., R.L-E. Methodology: E.M-S., J.S.O. Software: R.S.M., M.L.P.L. Validation: E.M-S., R.L-E. Formal analysis: M.E.L-N., R.R.N., L.S.B., C.J.B.O., D.M.P., L.P.S., G.M.L., D.G.S. Investigation: M.E.L. Resources: E.M-S., R.L-E. Data curation: M.E.L-N. Writing-original draft: M.E.L-N. Writing-review & editing: F.N.S.S. Visualization: E.M-S. Supervision: R.L-E. Project administration: E.M-S., R.L-E. Funding acquisition: E.M-S. All co-authors reviewed the final version and approved the manuscript before submission.

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