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Isolation of Acetic Acid-Producing Bacterial Strains and Utilization as Microbial Inoculants in Sorghum Silages

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Abstract: This study aimed to isolate, characterize, and identify acetic acid-producing lactic acid bacteria from fresh sorghum plants and silage, and to evaluate the effect of the isolates as microbial inoculants on taxonomic diversity and silage fermentation quality. For the first experimental stage, eight experimental silos were prepared, and the fresh sorghum plant cv. BRS Ponta Negra (*Sorghum bicolor* (L.) Moench.) was sampled to characterize and identify the bacteria. Five strains were chosen to be inoculated in the second experimental stage, in a 7 × 2 factorial design, with seven treatments and two opening times, in four replications. Four types of species were identified, with *Lactiplantibacillus plantarum* predominating at 72.73%. There was an interaction effect between treatments and opening times on effluent losses, gas losses, the population of lactic acid bacteria, yeasts, and lactic acid content. The aerobic stability treatments that stood out were *Lactiplantibacillus plantarum* (GML 66) and *Weissella cibaria*, which showed 71.75 and 68.87 h of stability. The use of *Lactiplantibacillus plantarum* (GML 66) as a microbial inoculant in sorghum silage increased the dry matter content, reduced effluent losses, and improved dry matter recovery. It also reduced the yeast population in the silage, promoting greater aerobic stability in the silage.

Keywords: acetic acid; aerobic stability; forage conservation; *Lactiplantibacillus plantarum*; fermentation quality

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1. Introduction

Forage sorghum has a fermentation profile considered suitable for the ensiling process, where it undergoes lactic fermentation. However, the major challenge in research involving silage from these forage plants is after the silo has been opened, due to its low aerobic stability associated with a high content of lactic acid and residual soluble carbohydrates and a low concentration of fermentation end products with antifungal capacity [1].

Therefore, despite the rapid acidification of the medium and the greater production of lactic acid during the fermentation period, lactic acid alone cannot inhibit the growth of molds and yeasts after the silo is opened. Molds and yeasts consume lactic acid and residual soluble carbohydrates and deteriorate the silage mass. At the same time, there is an increase in temperature, pH, CO_2 production, water, and heat [2]. As a result, due to the increase in pH, the action of other undesirable microorganisms, such as Enterobacteriaceae, leads to aerobic deterioration of the silage.

It is therefore very difficult to control the epiphytic population of microorganisms present in the forage plant at the time of ensiling. Therefore, strains of heterofermentative lactic acid bacteria have been used as microbial inoculants to increase the aerobic stability of silage by producing organic acids with high antifungal power, such as acetic and propionic acid.

Some strains of heterofermentative lactic acid bacteria, such as *Lentilactobacillus buchneri*, can convert lactic acid into acetic acid and 1,2-propanediol during the fermentation phase of the ensiling process [3]. However, studies carried out using obligate and facultative heterofermentative *Lentilactobacillus* strains as a microbial additive in forage sorghum silages in tropical and semi-arid conditions remain inconclusive [4–12]. These results may be associated with the epiphytic populations of microorganisms found on plants grown in tropical and semi-arid climates.

The colonization of plant surfaces by epiphytic bacteria depends on many factors, including plant species in the region, climate, soil, vegetative stage of the plant, geographical location, intensity of solar radiation, and the type of fertilizer used [13,14].Ding et al. [15], evaluated the epiphytic bacterial community of the plant *in natura* and the dynamics of the bacterial community during the fermentation process of *Elymus nutans* silage grown in four different regions of the Qinghai-Tibet plateau. The authors observed that silage fermentation quality varied according to the areas where *Elymus nutans* was grown and the changes in bacterial diversity during the fermentation process were due to the epiphytic bacteria of *Elymus nutans*.

Therefore, selective cultivation and prospecting of lactic acid bacteria allows us to understand the dynamics of the dominant species in the plant and at each stage of the ensiling process, allowing us to select those that are best adapted to be used as microbial inoculants. Studies of this nature are incipient with some forage plants, and in the semi-arid regions, there are no records in the literature of studies of this nature.

Thus, lactic acid bacteria capable of converting lactic acid into acetic acid and 1,2-propanediol isolated from forage sorghum grown in semi-arid climates can be used as more competitive inoculants than the commercial strains found on the market. Therefore, this study aimed to isolate, characterize, and identify acetic acid-producing lactic acid bacteria from fresh and ensiled forage sorghum grown in a semi-arid region, as well as to evaluate the effects of applying the isolates as microbial inoculants on taxonomic diversity, fermentation quality, losses during the ensiling process, chemical composition and aerobic stability of forage sorghum silages.

2. Materials and Methods

The flowchart of the activities carried out during the research is shown in Figure 1.

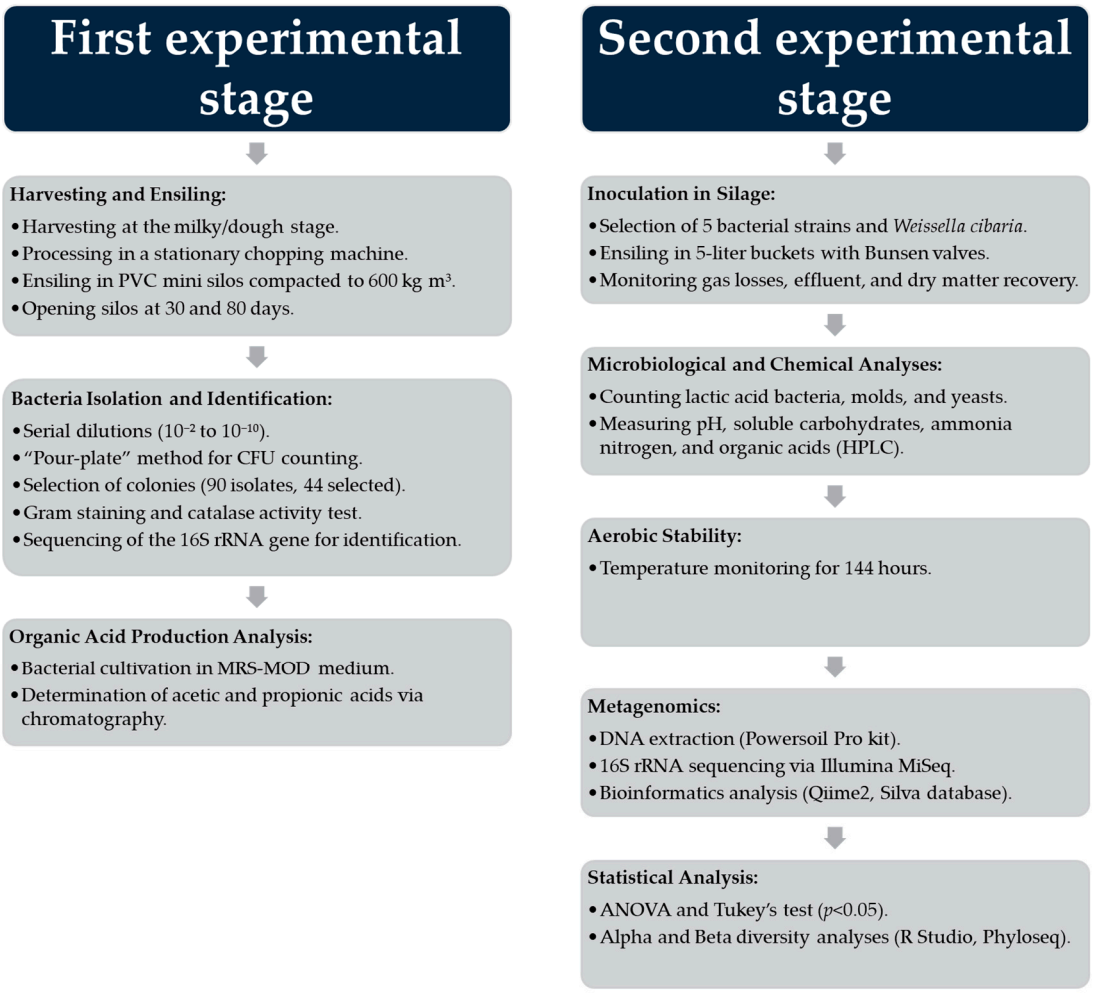

Figure 1. Flowchart of the experimental stages.

2.1. Site and Treatments

The first trial was conducted between September and December 2021, in the Forage Crop Laboratory owned by the Agriculture Sciences Center of the Federal University of Paraíba, Areia, PB, which is inserted in the micro-region of Brejo Paraibano, located at the geographic coordinates 6°58′12″ South latitude, 35°42′15″ West Greenwich longitude, at 619 m of altitude.

The sorghum was planted on a private property, located at 7°23′26″ South latitude, 36°48′30″ West longitude, and 529 m of altitude, in the town of São José dos Cordeiros, mesoregion of Borborema, and in the microregion of Western Cariri, known as Cariri Paraibano. It presents a Bsh climate (hot semi-arid), according to the Köppen classification, with rainfall from February to June, annual precipitation and average temperature around 551.7 mm and 23 °C, respectively. After the analysis, the soil was corrected to meet the needs of the crop and sown manually, using seeds of sorghum cv. BRS Ponta Negra (*Sorghum bicolor* (L.) Moench.) in sucrose at a depth of approximately 1.0 cm in an area of approximately 0.5 ha, with plant spacing of 0.7 m between rows totaling 10 plants per linear meter, and harvested when the grains reached the milky/dough stage.

After harvest, the sorghum was processed in a stationary chopping machine and then ensiled in experimental polyvinyl chloride (PVC) mini silos measuring 15 cm in diameter × 30 cm in height. The material was compacted with the aid of wooden sticks until reaching a density of 600 kg m^3 on a fed basis (FB) in each mini silo. During the

process, representative samples of the *in natura* plant were collected and then 8 experimental silos were constructed and opened at 30 and 80 days after ensiling.

2.2. Cultivation, Isolation, Characterization, and Identification of Bacteria

Strains of lactic acid fermenting bacteria were isolated from samples of the sorghum plant grown in the semi-arid region and throughout the two opening times. A sample of the material was used for the subsequent dilutions (10^{-2} to 10^{-10}), which were used for plating by the "pour–plate" method, and the plates with values between 30 and 300 colony forming units (CFU) were considered countable and isolable [16].

To test the use of anaerobic lactic acid, bacteria were grown according to the methodology described by Oude Elferink et al. [17], adopting modified MRS-Broth medium (MRS-MOD medium) with the following composition (per L of distilled water): peptone (5.0 g), yeast extract (2.0 g), Tween 80 (0.5 mL), potassium phosphate—K_2HPO_4 (1.0 g), monosodium phosphate—$NaH_2PO_4 \cdot H_2O$ (3.0 g), sodium acetate (0.6 g), magnesium sulfate—$MgSO_4$ $7H_2O$ (0.2 g), manganese sulfate—$MnSO_4$-H_2O (0.04 g), agar (15 g), lactic acid (4.8 mL), glucose (1 g) and acetic acid (1.5 mL).

The isolates were collected from the plants before ensiling and at each silo opening time (30 and 80 days). Colonies were selected from the plates of the highest dilutions, adopting the selection criterion of the square root of the total count of each plate selected. Ninety colonies were selected from the different plates and opening times.

After selecting the colonies, 44 were chosen and subjected to the Gram stain and catalase activity procedure, identified according to their origin. After that, the Gram-positive and catalase-negative bacteria were cultured in test tubes containing sterile AGAR MRS medium and sent to GoGenetic—Curitiba/PR, responsible for the extraction of DNA from the isolates, which were evaluated by Sanger sequencing of the coding region of the 16S rRNA, comparing the sequences obtained from each isolate with the sequences available in the GenBank database. The 16S rRNA gene sequences that showed similarity equal to or greater than 97% were considered as belonging to the same Operational Taxonomic Unit (OTU) [18].

A 2 mL aliquot of the isolated bacteria culture broth was acidified with 0.15 mL of 50% sulfuric acid solution, three days after growth in broth, and sent to the Instrumental Chromatography Laboratory of the Chemistry Department of the Federal University of Pernambuco–Recife, Pernambuco, to determine the organic acid contents (acetic and propionic acid) [19].

2.3. Use of Acetic-Producing Bacteria as Inoculants in Sorghum Silage

After selective cultivation, identification, and evaluation of organic acid production, five bacterial strains were chosen based on acetic acid production to be used as microbial inoculants in the second experimental stage, which was conducted between February and May 2022 in the Forage Crop Laboratory owned by the Agriculture Sciences Center of the Federal University of Paraiba, Areia, PB, inserted in the micro-region of Brejo Paraibano, located at the geographic coordinates 6°58′12″ South latitude, 35°42′15″ West Greenwich longitude and 619 m of altitude. Sowing was performed in the same property, following the same procedures as the execution of the first experimental stage, ensuring that sorghum with the same environmental conditions of origin was used.

The experimental design was completely randomized, arranged in a 7 × 2 factorial scheme, with 7 treatments and 2 opening times (30 and 80 days), in 4 replications, totaling 56 experimental silos. Out of the treatments, five were the strains of lactic acid bacteria isolated from the *in natura* sorghum plant and silage from the first experimental stage,

based on the production of acetic acid, in addition to the control treatment—no inoculant, and the treatment with *Weissella cibaria*.

Weissella cibaria was isolated from cactus pear by Pereira et al. [20], and has been reported to have positive effects on the aerobic stability of cactus pear and millet silages [21]. Thus, it was used as a positive control.

Therefore, the treatments were:

1. Control—no inoculant.
2. GML 09—*Lactiplantibacillus plantarum*.
3. GML 11—*Pediococcus pentosaceus*.
4. GML 51—*Lactiplantibacillus plantarum*.
5. GML 66—*Lactiplantibacillus plantarum*.
6. GML 68—*Lactiplantibacillus plantarum*.
7. *Weissella cibaria*.

Before ensiling, the five isolated strains as well as *Weissella cibaria* were incubated in MRS broth at 37 °C in three successive activations every 24 h. Dilutions were adjusted aiming to apply 10^{-6} colony-forming units per gram of forage silage. In the control treatment—no inoculation, the same amount of distilled water was added as in the inoculant mixture to remove the effect of adding the inoculant solution. The chemical and microbiological composition of the treatments on the day of ensiling can be seen in Table 1.

Table 1. Chemical composition and microbial counts in sorghum silage added with facultative heterofermentative bacteria.

Inoculant	g kg^{-1}					pH [6]	BC [7]
	DM [1]	MM [2]	CP [3]	EE [4]	CHOs [5]		
Control	292.10	102.30	71.70	16.8	134.4	4.98	0.19
L.p.[8] (GML 09)	300.40	78.60	53.30	16.4	108.3	5.25	0.15
P.p.[9] (GML 11)	315.50	92.10	61.90	17.6	128.3	5.35	0.12
L.p.[8] (GML 51)	296.00	79.40	64.50	19.9	141	5.3	0.16
L.p.[8] (GML 66)	306.90	82.30	61.20	21.7	128.7	5.2	0.17
L.p.[8] (GML 68)	304.40	76.20	59.80	19.2	118.5	4.76	0.17
W. cibaria	292.80	79.70	68.30	15.2	150.2	5.26	0.17
Inoculant	**CFU g^{-1} of Silage**						
	LAB [10]		**MOLD [11]**		**YEA [12]**		
Control	6.20		5.84		6.44		
L.p. (GML 09)	6.48		6.27		6.18		
P.p. (GML 11)	6.59		6.09		6.28		
L.p.[8] (GML 51)	6.41		6.81		6.35		
L.p.[8] (GML 66)	6.25		6.10		6.23		
L.p.[8] (GML 68)	6.80		6.39		6.80		
W. cibaria	6.59		6.21		6.16		

[1] DM: dry matter; [2] MM: mineral matter; [3] CP: crude protein; [4] EE: ether extract; [5] CHOs: soluble carbohydrates; [6] pH: hydrogen potential; [7] BC: buffer capacity; [8] *L.p.*: *Lactiplantibacillus plantarum*; [9] *P.p.*: *Pediococcus pentosaceus*; [10] LAB: lactic acid bacteria; [11] MOLD: molds; [12] YEA: yeasts.

The ensiling was performed in 5 L capacity buckets, compacted the density of 600 kg m^3, equipped with a Bunsen valve for gas exhaust, adding to the bottom of the silos one kilogram of fine dry sand, separated from the ensiled material by a non-woven fabric, allowing the capture and quantification of the effluent produced by the silage.

Selective culture media for each microbial group was used to quantify the microbial populations. Ten grams of fresh silage were weighed and added to 90 mL of distilled water with manual shaking followed by serial dilutions ranging from 10^{-2} to 10^{-7}. After

that, the plating of each experimental replication was performed in duplicate for each culture medium. For the cultivation of lactic acid bacteria (LAB), Man, Rogosa, and Sharpe medium (MRS Agar) was used and incubated for 48 h in biochemical oxygen demand (BOD) at 35 °C; for the molds and yeasts (YEA) populations, potato dextrose agar (PDA) was used, acidified with 1% tartaric acid at 10%, after 72 h, both incubated in BOD at 30 °C. After the incubation period for each microbial population, the plates with CFU ranging from 30 to 300 were counted, according to Kung Jr. [16], differentiating the colonies of molds and yeasts by morphological characteristics.

The pH, buffer capacity, and soluble carbohydrates were measured in the samples of treatments before ensiling, while for the samples of the opening times, the other analyses described later were performed. The pH values were measured using a pH meter according to the methodology described by Bolsen et al. [22]. Ammonia nitrogen (NH_3-N) levels in silages were determined according to the methodology described by Chaney et al. [23]. The buffer capacity (BC) was determined according to the methodology proposed by Playne and McDonald [24], adapted by Mizubuti et al. [25]. Soluble carbohydrate contents were determined according to the methodology of DuBois et al. [26]. For the quantification of lactic acid (LA), acetic acid (AA), and propionic acid (PA) levels in the silage, high-performance liquid chromatography (HPLC), model SPD-10A VP, coupled with an ultraviolet (UV) detector, was employed. The analysis was performed at a wavelength of 210 nm, with a column flow rate of 0.6 mL/min, a column pressure of 87 kgf, and an injection volume of 10 µL. The samples were prepared using 10 g of material, diluted in 90 mL of distilled water, and subsequently filtered with Whatman filter paper prior to analysis [19].

To determine gas losses, effluent losses, and dry matter recovery, the experimental silos were weighed, accounting for their weight, according to Jobim et al. [27] using the following equations:

$$\text{GL: } (\text{SWc} - \text{SWo})/(\text{FMc} \times \text{DMc}) \times 100, \quad (1)$$

where GL = gas loss (%DM); SWc = silo weight at ensiling (closed) (kg); SWo = silo weight at opening (closed); FMc = forage mass at ensiling (kg); DMc = forage dry matter at ensiling (%).

$$\text{EL: } (\text{SEWo} - \text{S}) - (\text{SEWc} - \text{S})/\text{FMc} \times 100, \quad (2)$$

where EL = effluent losses; SEWo = weight of empty silo + sand at opening (kg); SEWc = weight of empty silo + sand at ensiling (kg); S = weight of the silo (kg); FMc = forage mass at ensiling (kg).

$$\text{DMR: } (\text{FMo} \times \text{DMo})/(\text{FMc} \times \text{DMc}) \times 100, \quad (3)$$

where DMR = dry matter recovery (%); FMo = forage mass at opening (kg); DMo = DM content at opening (%); FMc = forage mass at ensiling (kg); DM = DM content at ensiling (%).

Chemical composition analyses were performed according to the methodologies described by AOAC [28] for the contents of dry matter (DM) (method 934.01), mineral matter (MM) (method 930.05), crude protein (CP) (method 920.87), and ether extract (EE) (method 920.39).

The evaluation of aerobic stability was performed from 2 kg of representative samples of the silages, which were packed again in the same experimental silos with no compaction, for 144 h. A thermometer was inserted in the geometric center of each experimental silo to monitor the temperature every thirty minutes, controlling the room temperature to 25 °C, and defining the aerobic stability break, according to Kung Jr. and Ranjit [19], when the silage temperature exceeded 2 °C above the room temperature.

The metagenomic diversity evaluation was performed in the Laboratory of Products of Animal Origin, owned by the Agriculture Sciences Center of the Federal University of Paraiba. Three replications of each treatment were collected during ensiling and at the opening time of 80 days, being immediately stored at −80 °C for DNA sequencing of the 16S rRNA gene. For genomic DNA extraction, 25 g of each sample was diluted in 225 mL of sterile 0.85% saline solution (100 mL of distilled water to 0.85 g of NaCl P.A. solute), placed in an automatic homogenizer for 3 min, and the entire solution was filtered. The entire filtered solution was centrifuged (6000 spins for 10 min at 4 °C) in a Falcon tube, preserving the pellet and discarding all supernatant. Genomic DNA was extracted using a commercial kit (Powersoil Pro DNA, Qiagen, Hilden, Germany). DNA integrity was assessed by 1% agarose gel electrophoresis and quantified by fluorometry (Qubit, Thermo Fisher, Waltham, MA, USA). The V3–V4 regions of the microbial 16S rRNA gene were amplified by PCR (95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension to 72 °C for 5 min) using the primers: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGCAG-3′ and R: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATCC-3′. The amplicon library was prepared using the Nextera XT Index Kit Set A (Illumina Inc., San Diego, CA, USA) and magnetic beads were used for cleaning and purification (Agencourt AMPureXP, Beckman Coulter, Indianapolis, IN, USA). Paired-end sequencing was performed on the Illumina MiSeq platform using a 500-cycle V2 kit (Illumina Inc., San Diego, CA, USA).

The reads were processed using the Qiime2 platform. Forward and reverse sequences were imported joined and the reads with low quality were removed, applying the score Q > 20 as quality control through the Dada2 program. Chimeric and unclassified sequences were removed from the analyses. Taxonomic identifications were made using the Silva databases (https://www.arb-silva.de/ accessed on 19 March 2023).

2.4. Statistical Analysis

The results obtained were evaluated through analysis of variance to verify the significance of the effects of inoculant and opening time and the interaction between factors, and the means were compared by Tukey's test, adopting 0.05 as a critical probability level. The procedures were performed with the SAS 9.4 (SAS Institute, 2015, Cary, CN, USA) software. Data were analyzed using the following model:

$$Yijk = \mu + Ti + Aj + (T \times A)ij + \varepsilon ijk$$

where Yijk is the observation regarding the combination of lactic acid bacteria strains isolated and opening time, μ is the general average, Ti is the isolated effect of the isolated lactic acid bacteria strains, Aj is the isolated effect of opening time, (T × A)ij is the effect of the interaction between the strains of lactic acid bacteria isolated and the opening time, and εijk is the random error associated with the observation.

Alpha and Beta diversity analyses were performed using R Studio software version 4.2.2 (Posit PBC, Boston, MA, USA), with the Phyloseq analysis package (https://joey711.github.io/phyloseq/ accessed on 19 March 2023). Tukey's statistical tests were applied for the diversity indices, considering a p-value < 0.05 as statistical difference. Three samples per treatment were standardized based on rarefaction curve analysis and sequencing depth.

3. Results

3.1. Identification and Characterization of Bacteria

All isolated bacteria, whether originating from the *in natura* sorghum plant or silage, were classified as Gram-positive and catalase-negative (Table 2).

Table 2. Biochemical and morphological characteristics of isolates from *in natura* sorghum and silage.

Isolate	Origin	Form	Gram Staining	Catalase Activity
GML 08	Plant	*Bacillus*	+	−
GML 09	Plant	*Bacillus*	+	−
GML 11	Plant	*Bacillus*	+	−
GML 12	Plant	*Bacillus*	+	−
GML 14	Plant	*Bacillus*	+	−
GML 17	Plant	*Bacillus*	+	−
GML 19	Plant	*Bacillus*	+	−
GML 26	Plant	*Bacillus*	+	−
GML 27	Plant	*Bacillus*	+	−
GML 28	Plant	*Bacillus*	+	−
GML 32	Silage	*Bacillus*	+	−
GML 34	Silage	*Bacillus*	+	−
GML 35	Silage	*Bacillus*	+	−
GML 36	Silage	*Bacillus*	+	−
GML 39	Silage	*Bacillus*	+	−
GML 40	Silage	*Bacillus*	+	−
GML 43	Silage	*Bacillus*	+	−
GML 44	Silage	*Bacillus*	+	−
GML 45	Silage	*Bacillus*	+	−
GML 47	Silage	*Bacillus*	+	−
GML 49	Silage	*Bacillus*	+	−
GML 51	Silage	*Bacillus*	+	−
GML 54	Silage	*Bacillus*	+	−
GML 55	Silage	*Bacillus*	+	−
GML 57	Silage	*Bacillus*	+	−
GML 59	Silage	*Bacillus*	+	−
GML 60	Silage	*Bacillus*	+	−
GML 61	Silage	*Bacillus*	+	−
GML 63	Silage	*Bacillus*	+	−
GML 64	Silage	*Bacillus*	+	−
GML 65	Silage	*Bacillus*	+	−
GML 66	Silage	*Bacillus*	+	−
GML 68	Silage	*Bacillus*	+	−
GML 69	Silage	*Bacillus*	+	−
GML 70	Silage	*Bacillus*	+	−
GML 71	Silage	*Bacillus*	+	−
GML 72	Silage	*Bacillus*	+	−
GML 73	Silage	*Bacillus*	+	−
GML 74	Silage	*Bacillus*	+	−
GML 75	Silage	*Bacillus*	+	−
GML 76	Silage	*Bacillus*	+	−
GML 77	Silage	*Bacillus*	+	−
GML 78	Silage	*Bacillus*	+	−
GML 79	Silage	*Bacillus*	+	−

+: Gram positive; −: Catalase activity negative.

The concentrations of organic acids varied according to each strain, ranging from 9.93 to 40.41 g kg^{-1} of acetic acid and from 0 to 12.84 g kg^{-1} of propionic acid (Table 3).

Table 3. Content (in g kg^{-1}) of acetic acid (AA) and propionic acid (PA), as well as acetic acid/propionic acid ratio ($AA:PA^{-1}$) per LAB strain in MRS broth.

Isolate	AA [1]	PA [2]	AA/PA [3]
GML 08	19.23	0.12	159.09
GML 09	28.35	0.16	174.16
GML 11	33.93	0.06	533.67
GML 12	19.50	0.11	174.42
GML 14	24.07	0.05	502.93
GML 17	14.71	0.11	135.79
GML 19	21.34	0.03	636.33
GML 26	21.13	0.03	625.78
GML 27	23.94	0.08	291.85
GML 28	21.44	0.04	546.34
GML 32	9.93	0.10	99.90
GML 34	16.30	0.02	658.08
GML 35	17.45	0.03	555.91
GML 36	25.68	0.05	495.45
GML 39	17.04	0.04	448.91
GML 40	23.51	0.14	172.15
GML 43	20.47	0.03	642.69
GML 44	24.52	0.07	366.16
GML 45	21.26	0.07	294.15
GML 47	16.82	0.07	256.81
GML 49	22.52	0.07	341.98
GML 51	27.03	0.00	-
GML 54	21.18	0.00	-
GML 55	22.84	0.38	60.52
GML 57	21.86	0.20	106.89
GML 59	23.22	0.16	147.62
GML 60	20.95	0.45	46.50
GML 61	20.51	0.24	84.03
GML 63	23.64	0.12	200.54
GML 64	22.72	0.26	89.05
GML 65	23.64	0.44	53.46
GML 66	40.07	8.91	4.50
GML 68	40.41	12.84	3.15
GML 69	20.55	0.25	82.49
GML 70	14.63	0.55	26.55
GML 71	13.21	0.19	70.04
GML 72	16.61	1.03	16.20
GML 73	23.46	2.26	10.39
GML 74	24.69	2.81	8.79
GML 75	19.55	0.77	25.47
GML 76	24.05	1.63	14.77
GML 77	23.75	2.43	9.76
GML 78	24.14	3.19	7.56
GML 79	22.26	2.14	10.41

[1] AA: acetic acid; [2] PA: propionic acid; [3] AA/PA: acetic acid propionic acid ratio.

All isolated strains showed sequence similarity of base pairs equal to or greater than 99%, compared to the GenBank database through the BLAST algorithm (National Center for Biotechnology Information, Bethesda, MD, USA). Among the 44 isolates, four types of bacteria species were identified: *Lactiplantibacillus plantarum*, *Pediococcus pentosaceus*, *Lactiplantibacillus plantarum/pentosus*, and *Limosilactobacillus reuteri*. There was a predominance of *Lactiplantibacillus plantarum* among the 44 strains isolated, regardless of their origin (*in natura* sorghum plant or silage), corresponding to 72.73% of the total (Table 4).

Table 4. Isolates, bacterial species similarity and base number of isolates from *in natura* sorghum and silage.

Isolate	Species	Similarity (%)	Base
GML 08	*Lactiplantibacillus plantarum*	99	1418/1419
GML 09	*Lactiplantibacillus plantarum*	99	1368/1379
GML 11	*Pediococcus pentosaceus*	100	1101/1101
GML 12	*Pediococcus pentosaceus*	100	1338/1338
GML 14	*Pediococcus pentosaceus*	100	1352/1352
GML 17	*Pediococcus pentosaceus*	99	13,144/1349
GML 19	*Lactiplantibacillus plantarum*	99	1383/1394
GML 26	*Lactiplantibacillus plantarum*	99	1397/1405
GML 27	*Lactiplantibacillus plantarum*	99	1401/1409
GML 28	*Pediococcus pentosaceus*	100	1270/1270
GML 32	*Lactiplantibacillus plantarum*	99	1449/1454
GML 34	*Lactiplantibacillus plantarum*	99	1441/1443
GML 35	*Lactiplantibacillus plantarum*	99	1457/1465
GML 36	*Lactiplantibacillus plantarum*	99	1449/1454
GML 39	*Lactiplantibacillus plantarum*	99	1444/1453
GML 40	*Lactiplantibacillus plantarum*	99	1444/1450
GML 43	*Lactiplantibacillus plantarum*	99	1447/1453
GML 44	*Lactiplantibacillus plantarum*	99	1439/1451
GML 45	*Lactiplantibacillus plantarum*	99	1449/1454
GML 47	*Lactiplantibacillus plantarum*	99	1462/1472
GML 49	*Lactiplantibacillus plantarum*	99	1462/1473
GML 51	*Lactiplantibacillus plantarum*	99	1450/1456
GML 54	*Lactiplantibacillus plantarum*	99	1451/1456
GML 55	*Lactiplantibacillus plantarum*	99	1438/1440
GML 57	*Lactiplantibacillus plantarum*	99	1458/1465
GML 59	*Lactiplantibacillus plantarum*	99	1448/1456
GML 60	*Lactiplantibacillus plantarum*	99	1447/1453
GML 61	*Lactiplantibacillus plantarum*	100	899/899
GML 63	*Lactiplantibacillus plantarum/pentosus*	99	1018/1026
GML 64	*Lactiplantibacillus plantarum*	99	1155/1167
GML 65	*Lactiplantibacillus pentosus*	99	1315/1316
GML 66	*Lactiplantibacillus plantarum*	99	1203/1212
GML 68	*Lactiplantibacillus plantarum*	99	1310/1315
GML 69	*Lactiplantibacillus plantarum*	99	1311/1318
GML 70	*Lactiplantibacillus plantarum*	99	1065/1069
GML 71	*Lactiplantibacillus plantarum*	99	1425/1433
GML 72	*Lactiplantibacillus plantarum*	99	1314/1318
GML 73	*Lactiplantibacillus plantarum/pentosus*	99	1318/1319
GML 74	*Limosilactobacillus reuteri*	100	1310/1310
GML 75	*Lactiplantibacillus plantarum/pentosus*	99	1326/1333
GML 76	*Lactiplantibacillus plantarum/pentosus*	99	887/888
GML 77	*Lactiplantibacillus plantarum*	99	1321/1335
GML 78	*Lactiplantibacillus plantarum*	99	1343/1345
GML 79	*Lactiplantibacillus plantarum/pentosus*	99	840/842

The strains identified from GML 08 to GML 28 were isolated from *in natura* sorghum plants. The strains with identification from GML 32 to GML 79 were isolated from sorghum silage.

3.2. Use of Bacteria as Inoculants in Sorghum Silage

An interaction effect ($p < 0.05$) was observed for the DM ($p = 0.0001$) and CP ($p = 0.0004$) contents of the silages, depending on the use of strains as microbial inoculants and the opening periods of the experimental silos. There was a difference ($p < 0.05$) for DM content, expressed in g kg^{-1}, with the highest value observed at 30 days in the silage inoculated with strain GML 11 and the lowest in the silage inoculated with *W. cibaria*. At 80 days after ensiling, the highest DM content was observed in the control silage and the lowest in the silage inoculated with strain GML 68 (Table 5).

Table 5. Chemical composition of dry matter and nutrients (on a dry matter basis) of sorghum silages with microbial inoculants at two opening times.

Treatment	Ensiling Time	g kg^{-1}			
		DM [1]	MM [2]	CP [3]	EE [4]
Control	30 days [5]	277.0 [ab]	95.8	67.1 [a]	18.7
	80 days [6]	278.7 [a]	91.6	62.7 [b]	20.7
L.p. [7] (GML 09)	30 days [5]	278.1 [ab]	88.8	51.5 [b]	18.1
	80 days [6]	278.4 [ab]	87.1	72.9 [a]	18.4
P.p. [8] (GML 11)	30 days [5]	287.9 [a]	92.5	70.8 [a]	18.3
	80 days [6]	269.2 [ab]	92.4	72.2 [a]	18.5
L.p. [7] (GML 51)	30 days [5]	283.2 [ab]	90.6	73.2 [a]	20.7
	80 days [6]	276.1 [ab]	88.1	69.3 [a]	19.2
L.p. [7] (GML 66)	30 days [5]	272.7 [bc]	84.3	64.2 [ab]	21.1
	80 days [6]	274.2 [ab]	83.4	64.5 [ab]	19.0
L.p. [7] (GML 68)	30 days [5]	271.8 [bc]	81.1	62.3 [ab]	19.2
	80 days [6]	267.6 [b]	82.4	71.8 [a]	19.0
W. cibaria	30 days [5]	266.0 [c]	82.9	60.9 [ab]	19.5
	80 days [6]	268.8 [ab]	77.2	74.91 [a]	19.2
SEM		0.22	0.19	0.30	0.80
		p-value			
Treatment		0.0001	0.0001	0.0274	0.2815
Ensiling time		0.0070	0.0759	0.0014	0.6247
Treatment × Ensiling time		0.0001	0.6727	0.0004	0.3821

SEM: standard error of the mean. Means followed by different letters in the column differ ($p < 0.05$) according to the Tukey test. [1] DM: dry matter; [2] MM: mineral matter; [3] CP: crude protein; [4] EE: ether extract; [5] 30 days: first opening time; [6] 80 days: second opening time; [7] *L.p.*: *Lactiplantibacillus plantarum*; [8] *P.p.*: *Pediococcus pentosaceus*.

A difference ($p < 0.05$) was observed for CP content, expressed in g kg^{-1} of DM, with the highest contents observed at 30 days in the control silages and those inoculated with strains GML 11 and GML 51, with no differences among them, and the lowest in the silage inoculated with strain GML 09. At 80 days after ensiling, the highest CP contents were observed in silages inoculated with strains GML 09, GML 11, GML 51, GML 68, and *W. cibaria*, with no differences among them, and the lowest content in the control silage (Table 5).

No interaction effect ($p > 0.05$) was observed for the ash ($p = 0.6727$) and EE ($p = 0.3821$) contents. However, an isolated strain effect was observed for ash content, expressed in g kg^{-1} of DM ($p = 0.0001$), with the highest contents observed in control silage and silage inoculated with strain GML 11, with no differences between them, and the lowest in silage inoculated with *W. cibaria*. However, no difference ($p > 0.05$) was observed for the EE contents ($p = 0.2815$) of the silages, with a general average of 19.28 g kg^{-1} of DM.

Regarding the opening periods after ensiling, no difference ($p > 0.05$) was found for the ash ($p = 0.0759$) or EE ($p = 0.6247$) variables, with overall averages of 87.00 and 19.25 g kg^{-1} of DM, respectively (Table 5).

There was an interaction ($p < 0.05$) between strains used as microbial inoculants and the opening periods of the experimental silos for EL ($p = 0.001$) and GL ($p = 0.0004$). A difference ($p < 0.05$) was observed for EL, expressed in kg t^{-1}, with the highest EL observed at 30 days in the silage inoculated with strain GML 68 and the lowest in the non-inoculated (control) silage and silages inoculated with strains GML 11, GML 51, and *W. cibaria*, with no differences among them (Table 6).

Table 6. Dry matter and nutrient recovery (DMR) and losses of sorghum silages with microbial inoculants at two opening times.

Treatment	Ensiling Time	DMR [1] (%)	EL [2] (Kg t^{-1})	GL [3] (% DM)
Control	30 days [4]	89.74	12.45 [b]	6.44 [a]
	80 days [5]	89.80	4.73 [b]	6.28 [a]
L.p. [6] (GML 09)	30 days [4]	91.54	14.57 [ab]	5.78 [a]
	80 days [5]	88.61	7.42 [a]	0.67 [b]
P.p. [7] (GML 11)	30 days [4]	91.77	11.67 [b]	7.18 [a]
	80 days [5]	85.61	7.67 [a]	0.50 [b]
L.p. [6] (GML 51)	30 days [4]	93.85	11.23 [b]	1.41 [b]
	80 days [5]	93.68	7.53 [a]	0.48 [b]
L.p. [6] (GML 66)	30 days [4]	87.95	15.02 [ab]	6.78 [a]
	80 days [5]	87.05	5.37 [ab]	7.17 [a]
L.p. [6] (GML 68)	30 days [4]	90.21	17.77 [a]	8.60 [a]
	80 days [5]	86.54	6.40 [a]	7.21 [a]
W. cibária	30 days [4]	90.41	10.13 [b]	7.59 [a]
	80 days [5]	88.78	7.04 [a]	7.59 [a]
SEM		1.02	0.92	0.61
		p-value		
Treatment		0.0002	0.0058	0.0001
Ensiling time		0.0006	0.0001	0.0001
Treatment × Ensiling time		0.0799	0.0004	0.0001

Means followed by different letters in the column differ ($p < 0.05$) according to the Tukey test. SEM: standard error of the mean; [1] DMR: dry matter recovery; [2] EL: effluent losses; [3] GL: gas losses; [4] 30 days: first opening time; [5] 80 days: second opening time; [6] *L.p.*: *Lactiplantibacillus plantarum*; [7] *P.p.*: *Pediococcus pentosaceus*.

A difference ($p < 0.05$) was observed for GL, expressed in % DM, with the highest GL observed at 30 days in the control silage and silages inoculated with strains GML 09, GML 11, GML 66, GML 68, and *W. cibaria*, with no differences among them, and the lowest in silage inoculated with strain GML 51. At 80 days after ensiling, the highest GL values were observed in the control silage. Silages inoculated with strains GML 66, GML 68, and *W. cibaria*, had no differences among them, and the lowest values were observed in silages inoculated with strains GML 09, GML 11, and GML 51, with no differences among them (Table 6).

No interaction effect ($p = 0.0799$) was observed for the DMR of the silages. However, there was an isolated effect of the strains ($p = 0.0002$) used as microbial inoculants in the silages, with the highest DMR observed in silage inoculated with strain GML 51 and the lowest in control silage and silages inoculated with strains GML 09, GML 11, GML 66, GML 68, and *W. cibaria*, with no differences among them. Regarding the opening periods after

ensiling, a difference was observed ($p = 0.0006$), with the highest average observed at the 30 day opening period and the lowest at 80 days after ensiling (Table 6).

There was an interaction effect ($p < 0.05$) between the strains used as microbial inoculants and the opening periods of the experimental silos for BC ($p = 0.0006$). A difference ($p < 0.05$) was observed for BC (eq mg HCl 100 g^{-1} DM), with the highest averages observed at 30 days in silages inoculated with strains GML 66, GML 68, and *W. cibaria*, with no differences among them, and the lowest in the control silage and silages inoculated with strains GML 09, GML 11, and GML 51, with no differences among them. However, the treatment averages did not differ at 80 days after ensiling (Table 7).

Table 7. Fermentation profile of sorghum silages with microbial inoculants at two opening times.

Treatment	Ensiling Time	pH [1]	BC [2]	CHOs [3]	NH_3-N [4]
Control	30 days [5]	3.43	0.33 [b]	2.86	1.37
	80 days [6]	3.56	0.39 [a]	2.83	0.93
L.p.[7] (GML 09)	30 days [5]	3.41	0.33 [b]	2.63	3.77
	80 days [6]	3.43	0.36 [a]	3.10	0.91
P.p.[8] (GML 11)	30 days [5]	3.41	0.33 [b]	2.93	3.15
	80 days [6]	3.43	0.37 [a]	2.87	0.74
L.p.[7] (GML 51)	30 days [5]	3.47	0.32 [b]	2.99	0.70
	80 days [6]	3.43	0.36 [a]	2.75	1.33
L.p. [7] (GML 66)	30 days [5]	3.46	0.43 [a]	2.44	0.73
	80 days [6]	3.46	0.38 [a]	2.49	0.72
L.p. [7] (GML 68)	30 days [5]	3.39	0.43 [a]	2.01	0.74
	80 days [6]	3.45	0.38 [a]	2.48	0.70
W. cibaria	30 days [5]	3.35	0.41 [a]	2.44	2.65
	80 days [6]	3.42	0.39 [a]	2.83	0.71
SEM		0.02	0.01	0.66	0.71
		p-Value			
Treatment		0.0021	0.0001	0.2605	0.1871
Ensiling time		0.0084	0.1641	0.3708	0.0118
Treatment × Ensiling time		0.0890	0.0006	0.8487	0.1181

SEM: standard error of the mean. Means followed by different letters in the column differ ($p < 0.05$) according to the Tukey test. [1] pH: hydrogen potential; [2] BC: buffer capacity; [3] CHOs: soluble carbohydrates; [4] NH_3-N: ammonia nitrogen; [5] 30 days: first opening time; [6] 80 days: second opening time; [7] *L.p.*: *Lactiplantibacillus plantarum*; [8] *P.p.*: *Pediococcus pentosaceus*.

No interaction effect ($p > 0.05$) was observed for pH ($p = 0.0890$), CHOs ($p = 0.8487$), or NH_3-N ($p = 0.1181$). An isolated strain effect ($p < 0.05$) was observed for pH variables ($p = 0.0021$), with the highest average observed in the control silage and the lowest in the silage inoculated with *W. cibaria*. However, no difference ($p > 0.05$) was observed for CHOs ($p = 0.2605$) or NH_3-N ($p = 0.1871$), with general averages of 2.68 and 1.36, respectively (Table 7).

Regarding the opening periods after ensiling, a difference ($p < 0.05$) was found for pH ($p = 0.0084$) and NH_3-N ($p = 0.0118$), with the highest averages observed at the opening periods of 80 and 30 days and the lowest at 30 and 80 days after ensiling, respectively. However, there was no effect ($p = 0.3708$) of the opening periods for CHOs, with a general average of 2.68 g kg^{-1} DM (Table 7).

An interaction effect ($p < 0.05$) was observed for the populations of LAB ($p = 0.0001$) and YEA ($p = 0.0001$), depending on the use of strains as microbial inoculants and the opening periods of the experimental silos. A difference ($p < 0.05$) was observed for the LAB

population (CFU g of silage), with the highest populations observed at 30 days in silages inoculated with strains GML 09, GML 11, and GML 68, with no differences among them, and the lowest in the control silages and those inoculated with strain GML 66. At 80 days after ensiling, the highest LAB population was observed in the silage inoculated with *W. cibaria* and the lowest in the control silage (Table 8).

Table 8. Microbial populations of sorghum silage with microbial inoculants at two opening times.

Inoculant	Ensiling Time	CFU g^{-1} of Silage		
		LAB [1]	MOLD	YEA [2]
Control	30 days [3]	4.67 [b]	4.42	4.41 [bc]
	80 days [4]	3.37 [c]	3.77	2.42 [c]
L.p.[5] (GML 09)	30 days [3]	5.37 [a]	4.90	4.96 [a]
	80 days [4]	4.40 [b]	4.27	3.90 [ab]
P.p.[6] (GML 11)	30 days [3]	5.16 [a]	4.74	4.91 [a]
	80 days [4]	4.33 [b]	4.20	4.03 [ab]
L.p.[5] (GML 51)	30 days [3]	4.85 [ab]	4.52	4.58 [ab]
	80 days [4]	4.49 [b]	4.32	4.21 [a]
L.p. [5] (GML 66)	30 days [3]	4.69 [b]	4.75	3.99 [c]
	80 days [4]	4.04 [b]	3.86	3.69 [b]
L.p. [5] (GML 68)	30 days [3]	5.00 [a]	4.73	4.53 [ab]
	80 days [4]	4.31 [b]	3.95	3.98 [ab]
W. cibaria	30 days [3]	4.92 [ab]	4.63	4.82 [ab]
	80 days [4]	4.99 [a]	4.24	4.02 [ab]
SEM		0.09	0.12	0.09
		p-value		
Treatment		0.0001	0.0145	0.0001
Ensiling time		0.0001	0.0001	0.0001
Treatment × Ensiling time		0.0001	0.1177	0.0001

Means followed by different letters in the column differ ($p < 0.05$) according to the Tukey test. SEM: standard error of the mean; [1] LAB: lactic acid bacteria; [2] YEA: yeasts; [3] 30 days: first opening time; [4] 80 days: second opening time; [5] *L.p.*: *Lactiplantibacillus plantarum*; [6] *P.p.*: *Pediococcus pentosaceus*.

A difference ($p < 0.05$) was observed for the YEA population (CFU g of silage), with the highest populations observed at 30 days in silages inoculated with strains GML 09 and GML 11, with no differences among them, and the lowest in silage inoculated with strain GML 66. At 80 days after ensiling, the highest YEA population was observed in silage inoculated with strain GML 51 and the lowest in the control silage (Table 8).

No interaction effect ($p > 0.05$) was observed for the MOLD population ($p = 0.1177$) of the silages (CFU g of silage). However, an isolated strain effect ($p = 0.0145$) was observed, with the highest MOLD populations found in silages inoculated with strains GML 09 and GML 11 and the lowest in the control silage. Regarding the opening periods, a difference ($p = 0.0001$) was observed, with the highest population observed at the 30 day opening period and the lowest at 80 days after ensiling (Table 8).

There was an interaction ($p < 0.05$) between the strains used as microbial inoculants and the opening periods of the experimental silos for AS ($p = 0.0001$), expressed in hours. A difference ($p < 0.05$) was observed for AS of the silages, with the longest time observed at 30 days in silage inoculated with strain GML 66 and the shortest in the control silage and silage inoculated with strain GML 09. At 80 days after ensiling, the longest AS time was observed in the control silage, and the shortest in silages inoculated with strains GML 09 and GML 11 (Table 9).

Table 9. Aerobic stability time (AS) and maximum temperature (TMax) reached by sorghum silages with microbial inoculants at two opening times.

Inoculant	Ensiling Time	AS [1] (h)	TMax[2] (°C)
Control	30 days [3]	25.12 [e]	27.25
	80 days [4]	107.33 [a]	33.45
L.p. [5] (GML 09)	30 days [3]	24.25 [e]	34.50
	80 days [4]	51.53 [e]	34.60
P.p. [6] (GML 11)	30 days [3]	44.50 [d]	31.75
	80 days [4]	52.87 [e]	32.60
L.p. [5] (GML 51)	30 days [3]	49.87 [cd]	31.42
	80 days [4]	54.00 [de]	33.80
L.p. [5] (GML 66)	30 days [3]	79.12 [a]	31.02
	80 days [4]	64.37 [c]	36.30
L.p. [5] (GML 68)	30 days [3]	55.00 [bc]	32.25
	80 days [4]	64.25 [cd]	33.47
W. cibaria	30 days [3]	61.37 [b]	32.77
	80 days [4]	76.37 [b]	34.72
SEM		2.05	1.05
		p-value	
Treatment		0.0001	0.0144
Ensiling time		0.0001	0.0001
Treatment × Ensiling time		0.0001	0.0575

Means followed by different letters in the column differ ($p < 0.05$) according to the Tukey test. SEM: standard error of the mean; [1] AS: aerobic stability; [2] TMax: maximum temperature; [3] 30 days: first opening time; [4] 80 days: second opening time; [5] *L.p.*: *Lactiplantibacillus plantarum*; [6] *P.p.*: *Pediococcus pentosaceus*.

No interaction effect ($p > 0.05$) was observed for Tmax ($p = 0.0575$) of the silages (°C). However, there was an isolated strain effect ($p = 0.0144$) from microbial inoculants, with the highest temperatures observed in silages inoculated with strains GML 09, GML 66, and *W. cibaria*, and the lowest in the control silage. Regarding the opening periods, a difference ($p = 0.0001$) was observed, with the highest temperature recorded at the 80 day opening period and the lowest at 30 days after ensiling (Table 9).

There was an interaction ($p < 0.05$) between the strains used as microbial inoculants and the opening periods of the experimental silos for AL ($p = 0.0001$). A difference ($p < 0.05$) was observed in LA contents of the silages, with the highest concentration observed at 30 days in silage inoculated with *W. cibaria* and the lowest in the control silage and silage inoculated with strain GML 09. At 80 days after ensiling, the highest LA content was observed in silage inoculated with strain GML 11 and the lowest in the control silage (Table 10).

No interaction effect ($p > 0.05$) was observed for AA ($p = 0.1547$), PA ($p = 0.9235$), or the LA:AA ratio ($p = 0.3706$). An isolated strain effect ($p < 0.05$) was observed for the LA:AA ratio ($p = 0.0001$), with the highest ratio observed in silage inoculated with GML 09 and the lowest in the control silage. However, no difference ($p > 0.05$) was observed for AA ($p = 0.1931$) or PA ($p = 0.7711$), with general averages of 1.72 and 0.68 g kg, respectively (Table 10).

Regarding the opening periods after ensiling, a difference ($p < 0.05$) was observed for PA contents ($p = 0.0153$), with the highest concentration observed at the 30 day opening period and the lowest at 80 days after ensiling. However, no difference ($p > 0.05$) was

observed for AA (p = 0.7711) or the LA:AA ratio (p = 0.9401) across opening periods, with general averages of 1.72 and 3.34 g kg, respectively (Table 10).

Table 10. Organic acid production in sorghum silages with microbial inoculants at two opening times.

Inoculant	Ensiling Time	g kg^{-1}			
		LA [1]	AA [2]	PA [3]	LA:AA [4]
Control	30 days [5]	4.85 [d]	1.76	0.82	2.77
	80 days [6]	5.00 [c]	1.75	0.63	2.86
L.p. [7] (GML 09)	30 days [5]	6.00 [b]	1.62	0.74	3.75
	80 days [6]	5.71 [ab]	1.52	0.58	3.81
P.p. [8] (GML 11)	30 days [5]	5.51 [bc]	1.56	0.70	3.58
	80 days [6]	6.19 [a]	1.70	0.63	3.66
L.p. [7] (GML 51)	30 days [5]	5.38 [cd]	1.82	0.74	2.98
	80 days [6]	5.88 [ab]	1.81	0.55	3.29
L.p. [7] (GML 66)	30 days [5]	5.58 [bc]	1.64	0.86	3.49
	80 days [6]	5.84 [ab]	1.88	0.83	3.10
L.p. [7] (GML 68)	30 days [5]	6.11 [ab]	1.76	0.57	3.47
	80 days [6]	5.63 [ab]	1.70	0.52	3.31
W. cibaria	30 days [5]	6.64 [a]	1.95	0.78	3.40
	80 days [6]	5.43 [bc]	1.62	0.57	3.38
SEM		0.12	0.10	0.09	0.15
		p-Value			
Treatment		0.0001	0.1931	0.1322	0.0001
Ensiling time		0.3721	0.7711	0.0153	0.9401
Treatment × Ensiling time		0.0001	0.1547	0.9235	0.3706

Means followed by different letters in the column differ ($p < 0.05$) according to the Tukey test. SEM: standard error of the mean; [1] LA: lactic acid; [2] AA: acetic acid; [3] PA: propionic acid; [4] LA:AA: lactic acid: acetic acid ratio; [5] 30 days: first opening time; [6] 80 days: second opening time; [7] *L.p.*: *Lactiplantibacillus plantarum*; [8] *P.p.*: *Pediococcus pentosaceus*.

Regarding the Alpha diversity indices, there was a higher diversity for the treatment with *Weissella cibaria* when compared to the control treatment before ensiling, according to the Observed and Chao1 diversity indices, but there were no statistical differences between the communities for the Shannon and Simpson indices (Figure 2A). At the end of the 80 day fermentation period, no diversity differences were observed among the communities through the Alpha diversity indices (Figure 2B).

The Beta diversity index showed that before ensiling, the control treatment was more abundant, presenting a taxonomic composition more distinct from the other treatments (Figure 3A). The communities of the treatments did not present great differences in their composition after 80 days of fermentation when analyzed by the Beta diversity index (Figure 3B).

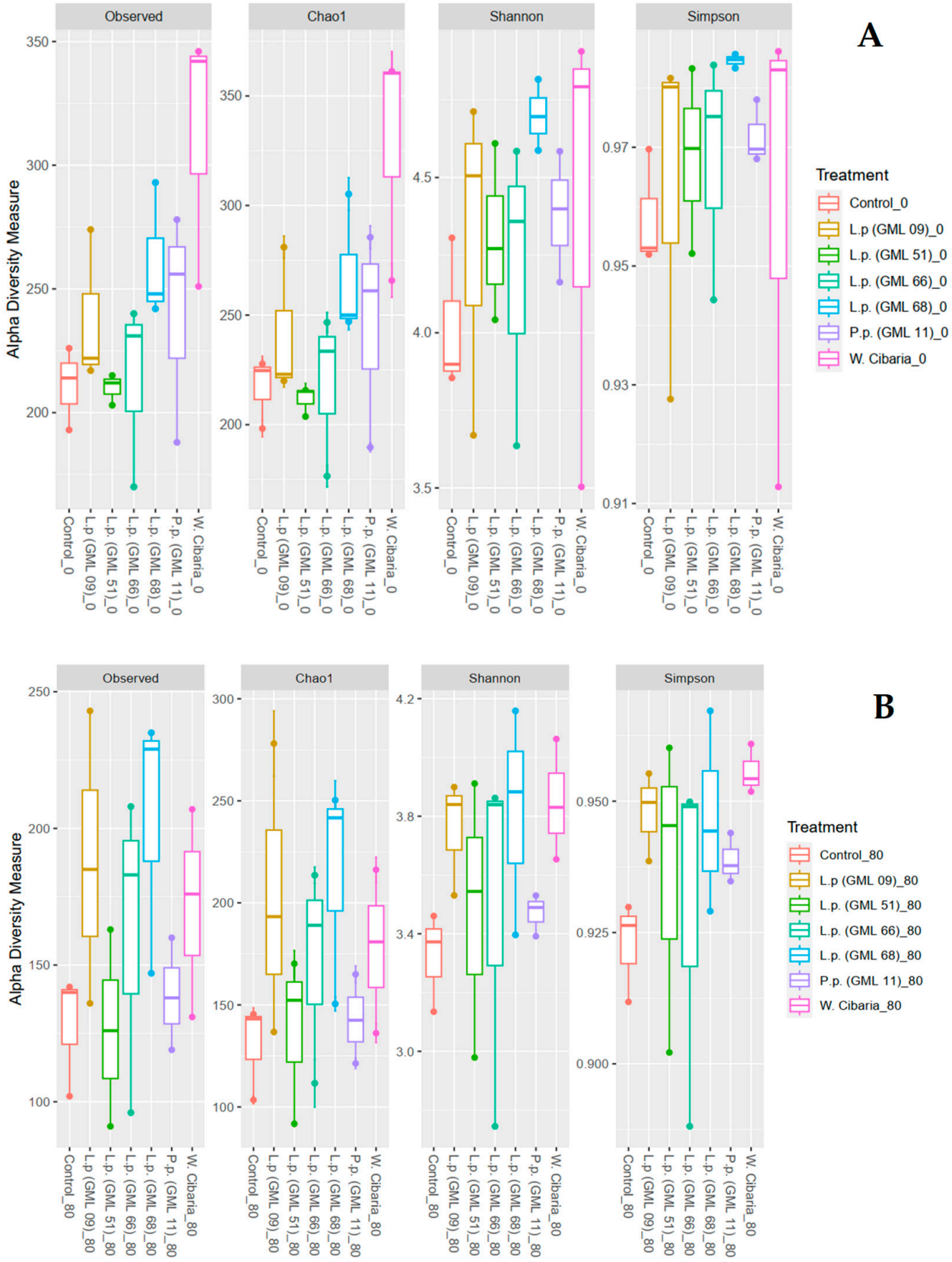

Figure 2. Alpha diversity analysis of metagenomes across all treatments at time zero (**A**) and 80 days after ensiling (**B**), based on Chao1, Shannon, and Simpson indices.

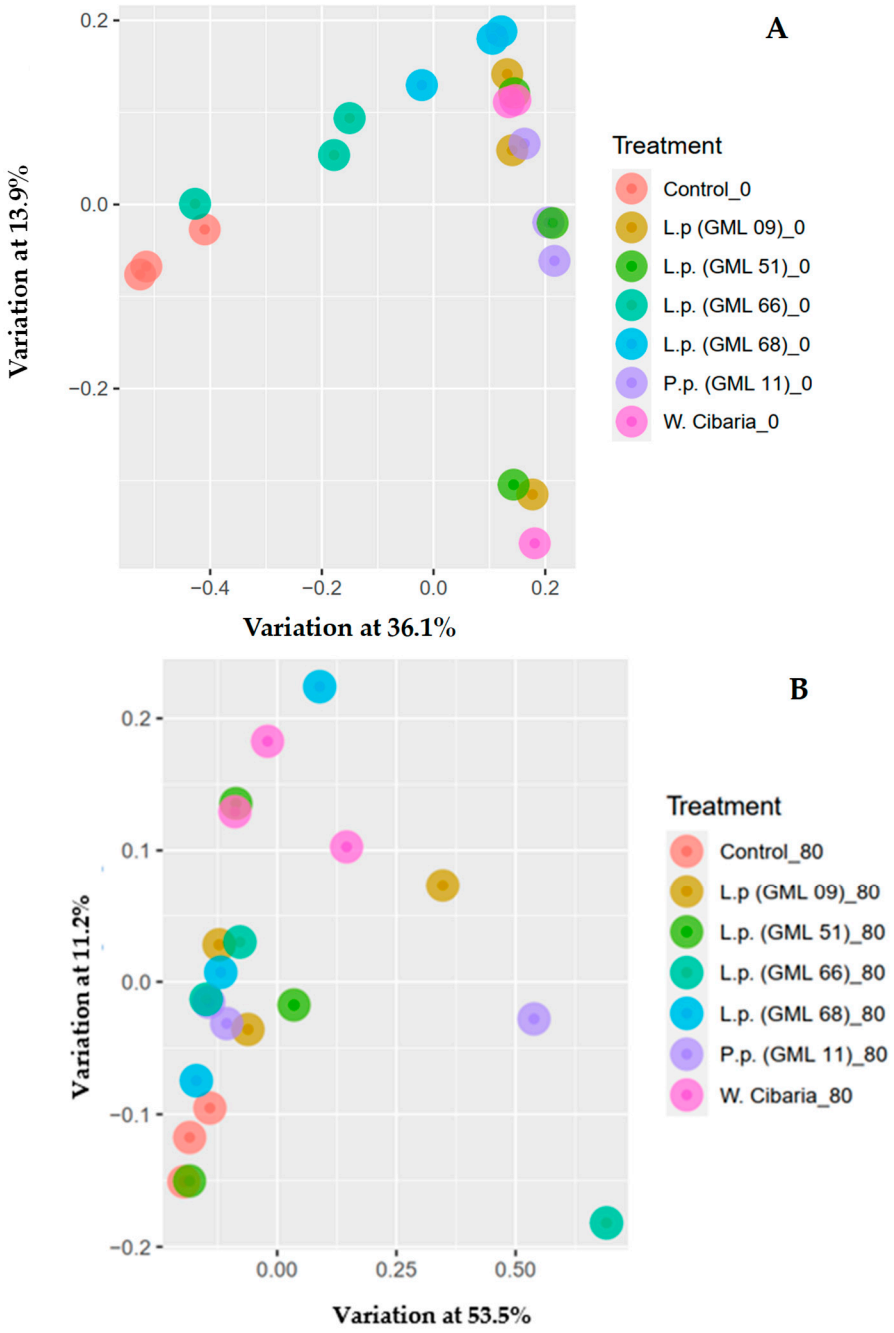

Figure 3. Beta diversity analysis of metagenomes across all treatments at time zero (**A**) and 80 days after ensiling (**B**), represented by principal coordinate analysis plots (PCoA).

4. Discussion

Lactic acid-producing bacteria are classified as Gram-positive, catalase-negative activity, non-spore-forming, lactic acid-producing, and facultative anaerobes (Zheng et al.) [29]. However, these bacteria can produce other types of organic acids besides lactic acid and can be characterized as homofermentative or heterofermentative.

The microorganisms identified in this study belong to the *Lactobacillaceae* family. This family of lactic acid-producing microorganisms has homofermentative and heterofermentative bacteria, as found by Zheng et al. [29], who stated that *Pediococcus* and the bacteria belonging to the *Lactiplantibacillus plantarum* group have an evolutionary link between them (Table 4). According to the same authors, despite having metabolic characteristics focused

on homofermentation, that is, lactic acid production, these bacteria are phylogenetically interconnected with heterofermentative bacteria.

The strains identified in this study produced acetic and propionic acid, highlighting *Lactiplantibacillus plantarum* (GML 66) and *Lactiplantibacillus plantarum* (GML 68) (Table 3). Although these bacteria are considered homofermentative, they were able to produce other organic acids, corroborating the claims of Zheng et al. [29], that they are phylogenetically interconnected with heterofermentative bacteria.

The observed DM, EE, and CP values (Table 5) are similar to the values found by Rodrigues et al. [30] and Santos et al. [31], who worked with sorghum silages with microbial inoculants. Studies of sugar sorghum bagasse silage with commercial microbial inoculants composed of *Lactiplantibacillus plantarum* and *Lentilactobacillus buchneri* obtained higher DM content in the treatments with these inoculants than the one without inoculation of bacteria [32], which corroborates the present study, where the treatments inoculated with *Lactiplantibacillus plantarum* (GML 09), *Pediococcus pentosaceus* (GML 11) and *Lactiplantibacillus plantarum* (GML 51) opened at 30 days remained statistically similar to the control treatment.

Only *Lactiplantibacillus plantarum* (GML 09) differed from the other treatments on the CP values (Table 5) in the shortest opening time. This result is associated with the NH_3-N value (Table 7) for the same treatment at the same opening time, indicating that there was a slightly more intense proteolytic activity than in the other treatments, thus resulting in a lower CP content (Table 5) than in the different treatments, and consequently, a higher NH_3-N content (Table 7) in this silage. According to Sun et al. [33] and Li et al. [34], the proteolytic activity that occurs in the fermentation process results in nitrogen compounds, amino acids, ammonia, and bioactive peptides, exhibiting probiotic characteristics related to *Lactiplantibacillus plantarum*.

A lower CP content (Table 5) was observed in the control silage compared to the other treatments, which differed at the longest opening time. This was directly influenced by the higher pH (3.56) (Table 7) at this same opening time, indicating that proteolysis may have occurred.

Inoculation with *Lactiplantibacillus plantarum* (GML 09) in the shortest opening time resulted in an unusual response, as indicated by Silva et al. [35], obtaining lower CP contents for inoculated sorghum and corn silages (54.8 and 70.0 g kg^{-1} DM, respectively) in comparison to the same silages without inoculation of microorganisms (55.8 and 72.2 g kg^{-1} DM, respectively). Michel et al. [36], studied inoculated sorghum silages and observed that inoculated silages obtained higher CP contents (67.7 g kg^{-1} DM) when compared to the silage without inoculants (64.767.7 g kg^{-1} DM), behaving similarly to the control treatment opened at 80 days of the present study (Table 5).

The silage inoculated with *Lactiplantibacillus plantarum* (GML 51) resulted in a higher DMR (Table 6), which was attributed to homolactic fermentation, producing much more lactic acid than acetic acid (Table 10). Despite the difference during the silage fermentation process, these changes were not enough to alter the DMR.

At 30 days after ensiling, silage inoculated with *W. cibaria* stood out from the others, obtaining the lowest EL among them (10.13 kg t^{-1}). In contrast, at 80 days after ensiling, the silages from the control treatment and *Lactiplantibacillus plantarum* (GML 66) showed the lowest EL, with 4.73 and 5.73 kg t^{-1}, respectively (Table 6). Dos Santos et al. [37], also found higher EL in corn silage without and with freeze-dried inoculant, whereas a lower EL (0.45 kg t^{-1}) was obtained when evaluating corn silage with the activated inoculant.

The inoculation with *Lactiplantibacillus plantarum* (GML 51) resulted in the lowest GL (1.41% DM) in the first opening. As for the second opening, the control, *Lactiplantibacillus*

plantarum (GML 66), *Lactiplantibacillus plantarum* (GML 68) and *Weissella cibaria* treatments resulted in the highest GL, ranging between 6.28 and 7.59% DM (Table 6).

Coelho et al. [38] observed that gas losses were not influenced by the effect of the joint inoculation of *Lactobacillus plantarum* and *Propionibacterium acidipropionici* in relocated corn silages. On the other hand, in the studies of Dos Santos et al. [37], GL was significantly different for silages with the active inoculant (5.55% DM), a result that was higher than that of control silages and silages with freeze-dried inoculant, which presented 2.67 and 3.17% DM, respectively.

For the data referring to silage BC (Table 7), the silages inoculated with *Lactiplantibacillus plantarum* (GML 66), *Lactiplantibacillus plantarum* (GML 68) and *Weissella cibaria* showed greater resistance among the other treatments in the first opening time, and no difference was observed among the treatments for the second opening time. Yin et al. [39] studied *Lentilactobacillus buchneri* in corn silages and observed that the inoculated silage obtained a higher concentration of total acids, although not very significant when compared to corn silage without inoculant. According to Yin et al. [39], chemical reactions can occur within the silo involving the organic acids produced, resulting in increased gas production and reduced buffer capacity. The treatments with *Lactiplantibacillus plantarum* (GML 66), *Lactiplantibacillus plantarum* (GML 68), and *Weissella cibaria* showed higher GL than the others in the first opening time, corroborating Yin et al. [39], who indicated that there was a chemical reaction decreasing the BC of these treatments for the next opening time.

A reduction was observed in all microbial populations (Table 8) at 80 days, in the fermentation stability phase. During this phase, it is observed that fermentation occurs more mildly (Table 10).

It was possible to see that in both opening times, the control treatment presented the lowest LAB counts. Because of the inoculation of bacteria in the other treatments, this result was already expected (Table 8). Rabelo et al. [40], Chen et al. [41] and Soundharrajan et al. [42] corroborated the LAB data of the present study, as in their studies higher LAB counts were also found in the silages inoculated with lactic acid bacteria when compared to the control treatment.

The same result was observed for the YEA population (Table 8). This may have happened due to the higher lactic acid production of the other treatments (Table 10), which can serve as the substrate for the development of YEA, as well as their lower pH values (Table 7), further favoring the growth of the YEA population (Table 8). Dos Santos et al. [37] also found similar results for the microbial population of YEA when opening the silos at 70 days, where the treatment with active inoculant obtained 5.17 CFU g^{-1} of silage, and the control treatment obtained 4.86 CFU g^{-1} of silage, which represents a decline of 0.31 CFU g^{-1} of silage.

Regarding the AS results among treatments, it was observed that *Lactiplantibacillus plantarum* (GML 09), *Pediococcus pentosaceus* (GML 11), *Lactiplantibacillus plantarum* (GML 51), and *Lactiplantibacillus plantarum* (GML 68) promoted reductions in the AS time of the silages, thus affecting them negatively. *Lactiplantibacillus plantarum* (GML 66) and *Weissella cibaria* prolonged the AS time of the silages.

It can be observed that there was an increase for AS and TMax to opening times when the silos were opened later. This increase means that, despite prolonging the stability time after opening the silos and exposure to oxygen, there was also an increase in the temperature of these silages during secondary microbial activity, raising their internal temperature.

When the silos were opened at 30 days, except for *Lactiplantibacillus plantarum* (GML 09) that was lower than the control treatment, all treatments obtained higher results, highlighting *Lactiplantibacillus plantarum* (GML 66). This prolonged stability is attributed to the microbiological data of the YEA population of this same treatment at the same opening

time (Table 8), and these microorganisms are responsible for promoting the deterioration of the silage, using both residual soluble carbohydrates and the lactic acid produced.

Regarding the second opening time, the best AS time was obtained by the control treatment, followed by *Weissella cibaria* and *Lactiplantibacillus plantarum* (GML 66). Despite being able to produce acetic acid in high amounts, the faster acidification of the medium and the higher lactic acid (Table 10) contents resulted in a higher YEA (Table 8) count in the inoculated silages over the 80 day period, where the control treatment stood out with the lowest YEA microbial population count for this opening time (Table 8). This result corroborates Ferrero et al. [43], who found an inversely proportional response between time to aerobic stability break and the yeast population.

Dos Santos et al. [37] observed a shorter AS time for the control treatment when compared to the inoculated sorghum silages. Nkosi et al. [44] evaluated silages inoculated with *Lactiplantibacillus plantarum* and *Lentilactobacillus buchneri* and observed that silages with *Lactiplantibacillus plantarum* obtained the worst AS, 46 h only, which was lower than the control treatment (53 h) and *Lentilactobacillus buchneri* (72 h). These data corroborate the present study for the results of the opening time of 80 days, since the strains used were *Lactiplantibacillus plantarum*, and it also obtained lower AS values when compared to the control. The study by Michel et al. [36] also corroborated these data, also observing better AS times for silages that were not inoculated. Thus, the isolates of the present study may be efficient in combinations with lactic acid heterofermentative bacteria, since alone they are not effective in controlling the aerobic stability of silages.

Commonly, acidic silages can promote the conversion of ethanol into acetic acid by the action of acetic bacteria, explaining what may have happened in the increase of AS time in this study (Table 9). From the short-term silo opening perspective, the silage without inoculation proved to be not very efficient, showing that inoculation is necessary in this case.

In both opening times, the silage with no microbial inoculant obtained the lowest lactic acid production values (Table 10). This result confirms the higher lactic acid production in the silages that were inoculated. Inoculation with *Weissella cibaria* resulted in higher lactic acid production, differing statistically only from the control treatment and from *Lactiplantibacillus plantarum* (GML 51). This result shows that the other strains inoculated in the silages, despite being considered homofermentative, behaved similarly to *Weissella cibaria*, which is heterofermentative.

In general, except for the control treatment, all inoculated silages obtained results between 4.36 CFU g^{-1} of silage (*Lactiplantibacillus plantarum* GML 66) and 4.95 CFU g^{-1} of silage (*Weissella cibaria*). These results are directly related to lactic acid production, which obtained the highest concentrations for silages with higher microbial counts of lactic acid bacteria (Table 10).

These data are also related to pH values (Table 7). According to Xu et al. [45], lactic acid bacteria can convert soluble carbohydrates into organic acids quickly, causing the pH to drop drastically to more acidic levels. This pH behavior occurred inversely to the production of lactic acid, since the increase in the concentration of organic acids, especially lactic acid, causes a more acidic pH in the silage (Tables 7 and 10). In addition, Dos Santos et al. [37] found the same inverse behavior for silages opened at 70 days, where the control silage and the silage inoculated with active inoculant obtained 46.61 and 38.56 g kg^{-1} DM, respectively, in which the control silage obtained a more acidic pH (3.52) and silage with active inoculant obtained a less acidic value (3.66), differing statistically from each other.

Between opening times, there was a higher production of propionic acid when the silo was opened sooner (Table 10). This result may be related to the higher availability of lactic

acid during the first opening period (Chen et al. [41]), serving as a substrate for the bacteria to metabolize and convert it into other acids, such as propionic acid.

The lactic acid:acetic acid ratio was directly affected by the difference between treatments for lactic acid production, since the greater the variation in one of the two acids, the greater the difference in the lactic acid:acetic acid ratio. Among the treatments, as the control obtained the lowest lactic acid production, it consequently obtained the lowest lactic acid:acetic acid ratio (Table 10).

The Alpha diversity data showed that there was a higher numerical concentration for the treatment with *Weissella cibaria* when compared to the control before the fermentation period (Figure 2A). After the fermentation period, there was a reduction of this bacterial population in the silages, so there was no difference between them. According to Du et al. [46], fermented silages have a lower Alpha diversity when compared to the same material before the fermentation period. This occurs due to the predominance of LABs, which cause a drop in the silage pH due to the production of organic acids, being able to inhibit and reduce the bacterial diversity present in the silage. As observed in Table 8, there was a reduction in all microbial populations quantified in this study (LABs, molds, and yeasts). These microbial population results are linked to the pH (Table 7) and organic acid (Table 10) values, confirming this reduction in Alpha diversity.

The Beta diversity results showed that the use of bacteria inoculation, regardless of the strain used, was able to promote differentiation among all treatments in comparison to the control before ensiling. After the fermentation period, no differences were observed among them (Figure 3B). This may have happened due to the greater abundance of some dominant bacterial species, being able to develop and grow in greater numbers, promoting a reduction in the diversification of the bacterial community present. Corroborating this study, Muraro et al. [47] also observed a reduction in Beta diversity, whose predominance occurred among the four main species, where the authors explained the fact that there were no significant differences between treatments due to the low diversity in this index.

Thus, it can be observed that the advance in the fermentation period of the silages, besides promoting a numerical reduction in the microbial population (also observed in the Alpha diversity) (Table 8 and Figure 2B), there is greater competition among dominant species until they reach their greatest abundance so that they reduce and even eliminate other less competitive species.

In sorghum silages, which are considered acidic silages, the transformation of ethanol by acetic bacteria can occur, being converted into acetic acid, explaining the increase in aerobic stability over time. However, when the silo is opened in a short time interval, with no use of microbial inoculants it may result in a less stable silage.

The inoculants *Lactiplantibacillus plantarum* (GML 66), *Lactiplantibacillus plantarum* (GML 68) and *Weissella cibaria* stood out among the strains used. Their performance was observed in both opening times, at 30 and 80 days of fermentation, which makes it possible to insert them in the composition of inoculants associated with each other or with other bacteria, such as *Lentilactobacillus buchneri*, capable of promoting greater dry matter recovery from silages, the production of organic acids and a greater durability of exposure to oxygen in a stable form of sorghum silages.

Thus, the strains *Lactiplantibacillus plantarum* (GML 66) and *Lactiplantibacillus plantarum* (GML 68) should be applied in future studies to validate their effectiveness in conjunction with other bacteria and in other forages.

5. Conclusions

The use of modified selective medium (MRS-MOD) and molecular techniques increases the accuracy of selecting new strains as microbial inoculants and consequently

enhances their effects on the fermentation process of silages. Furthermore, it will enable a more detailed characterization of the microbiota present in silages, facilitating the understanding of processes that are still poorly elucidated under tropical conditions.

Thus, the prospection of epiphytic bacteria from sorghum *in natura* and ensiled, in its different opening times, resulted almost entirely in the species *Lactiplantibacillus plantarum* and *Pediococcus pentaseus*. As the fermentation period progressed, the dominant species reached their greatest abundance, promoting a numerical reduction observed in the Alpha community. Despite this, *Lactiplantibacillus plantarum* (GML 66) had the best performance as an inoculant in sorghum silage.

Despite the advances achieved in the prospecting and isolation of new lactic acid bacteria strains from forage sorghum cultivated in arid and semi-arid regions, there are still limitations in capturing the full extent of microbial diversity present in the samples, particularly with regard to rare species, due to limited sequencing depth. In this context, future studies should be conducted with the aim of identifying new microbial strains, integrating complementary approaches such as metabolomic analyses to achieve a more comprehensive and functional characterization of the microbiome.

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