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ORIGINAL ARTICLE

Effects of *Lactobacillus buchneri* isolated from tropical maize silage on fermentation and aerobic stability of maize and sugarcane silages

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Abstract

This study was aimed to perform a screening of *Lactobacillus buchneri* strains from maize silage and use them as inoculant in maize and sugarcane silages. In all, 151 lactic acid bacteria (LAB) strains were isolated from whole-plant maize silage, and their identification was based on the sequence analysis of 16S rDNA. In total, 15 strains were categorized to the *L. buchneri* group and eight of these were selected based on growth rate and fermentation pattern. The selected strains were evaluated on fermentation and aerobic stability of maize and sugarcane silages. For maize, the inoculated silages had lower pH and higher LAB population, but lower acetic acid concentration in comparison with the untreated control silage. For sugarcane silage, the strains 56.1, 56.4 and 40788 resulted in highest dry-matter (DM) content and lowest DM losses. However, only the strain 40788 showed lowest counts of yeasts and moulds. Sugarcane silages inoculated with the strains 56.9, 56.26 and the untreated control silage showed highest concentrations of lactic acid and ethanol, besides the great DM losses. Even so, for both crops, the aerobic stability was not affected by inoculation. After air exposure, all silages increased temperature and had high population of yeast and moulds. Nevertheless, the strains 56.1 and 56.4 are promising for use as a silage inoculant.

KEYWORDS
16S rDNA, dry-matter loss, inoculant, lactic acid bacteria, *Saccharum officinarum*, *Zea mays*

1 | INTRODUCTION

The intensification of livestock systems to improve animal performance requires more feed with high quality and constant availability. In this context, silage is an important forage source in the tropics because of the dry season, which decreases the forage availability in the pastures (Pholsen, Khota, Pang, Higgs, & Cai, 2016). In addition, ensiling is the main method of forage preservation in tropical regions, since there are many difficulties to produce hay, due to high humidity and frequent rainfall at harvest period (Bernardes & Rêgo, 2014).

However, the hot weather also stimulates microbial growth and increases fermentation losses and aerobic spoilage of silage (Ashbell, Weinberg, Hen, & Filya, 2002). Furthermore, crops containing high concentration of starch or sugars, such as maize and sugarcane respectively, probably will have more yeasts and their silages are more susceptible to spoilage due to the large amount of substrates available for microbial activity (Kung, 2010).

Although the whole-plant maize is the most widely used crop as silage in the world, studies about microbial diversity of this silage in tropical climate are rare (Zhou, Drouin, & Lafrenière, 2016). Maize has adequate characteristics for good fermentation in a silo, but severe losses can occur during the feed-out phase (Santos et al., 2015). Sugarcane has high sugar content and low buffering capacity, which favour lactic acid production and fast pH drop, but normally had high

yeast population that leads to intense alcoholic fermentation and excessive dry-matter (DM) loss during ensilage and after air exposure, as well (Pedroso, Rodrigues, Barioni Júnior, & Souza, 2011).

Studies have shown that the *Lactobacillus buchneri* application in silages can reduce losses and increasing the aerobic stability, degradability rate and animal performance (Rabelo et al., 2017; Schmidt et al., 2014). This obligate heterolactic acid bacterium improves aerobic stability by increasing acetic acid concentration and decreasing moulds and yeasts of silages (Driehuis, Oude Elferink, & Spoelstra, 1999). However, there are few descriptions of the selection process of these bacterial strains.

Lactic acid bacteria (LAB) strains are frequently identified by sequencing the 16S region of rDNA because it is a rapid and reliable method to study biodiversity and population dynamics (Li & Nishino, 2011). In addition, the screening of LAB for use as silage inoculant based on growth rate and metabolite production has provided improvements in the silage quality (Ávila, Carvalho, Pinto, Duarte, & Schwan, 2014; Ni, Wang, Li, Cai, & Pang, 2015). Thus, the goals of the present study were to perform a screening of *L. buchneri* strains isolated from tropical maize silage and determine their effect on fermentation and aerobic stability of maize and sugarcane silages.

2 | MATERIALS AND METHODS

2.1 | Experimental area and climatic conditions

The experiments were conducted, and the crops were grown at the Department of Animal Science of the Federal University of Viçosa (Universidade Federal de Viçosa—UFV, Viçosa, MG, Brazil) between January and March 2014. Viçosa is located at 20°45′ South latitude, 42°51′ West longitude and 657 m above sea level with a mean annual rainfall of 1,341 mm. The regional climate is classified by Köppen standards as Cwb, i.e., winters are cold and dry, and summers can be hot and rainy (Sá Júnior, Carvalho, Silva, & Alves, 2012).

2.2 | Tropical maize silage characterization and LAB identification

Maize (*Zea mays* L.) plants were harvested when the kernels reached the hard dough stage (304 g DM/kg). Harvested material was chopped at 2 cm theoretical length of cut using a JF-92 Z10 forage harvester (JF Agricultural Machinery, SP, Brazil). Approximately, 500 g of chopped material was immediately conditioned in nylon-polyethylene bags (25 × 35 cm; Doug Care Equipment Inc., Springville, CA) to produce mini-silos, and the air was evacuated from the bags using a vacuum sealer (Eco vacuum 1040, Orved, Italy). The mini-silos were stored at room temperature and opened after 1, 3, 7, 14, 28 and 56 days of storage. Three replicates from the silage and fresh forage samples (total of 21 samples) were prepared. The samples were destined to determination of dry matter (at 105°C for 18 hr), and the water extract was prepared for chemical analysis and identification of LAB. The DM content was corrected for volatile compounds according to Weißbach and Strubelt (2008).

Water extracts from the silages and fresh forage samples were prepared by homogenizing 25 g of sample in 225 ml of sterile Ringer's solution (Oxoid, Hampshire, England) in an industrial blender for 1 min, and divided in two portions. One portion was subjected to serial dilutions ranging from 10^{-1} to 10^{-10} for microbial analysis (Table 1). Pour plates were prepared with de Man, Rogosa and Sharpe (MRS, Difco, São Paulo, Brazil) agar for LAB, and Potato Dextrose Agar (PDA; Difco, Sao Paulo, Brazil) containing 1.5% of tartaric acid solution (10% w./v.) for yeasts and moulds (Y&M). The MRS plates were incubated at 37°C for 48 hr in the anaerobic jars (Permution®, Curitiba, PR, Brazil). The PDA plates were incubated aerobically at 25°C for 5 days. All colonies were counted on plates with 25–250 well-isolated colony-forming units.

For the identification of LAB species, several colonies corresponding to the square root of the total colonies contained on the MRS agar plates were isolated at random (Holt, Krieg, & Sneath, 1994). The isolates were further purified by streaking individual colonies onto MRS agar containing bromocresol purple and $CaCO_3$ as indicators. All potential LAB were detected by a yellowish colony and a clear zone caused by the dissolution of $CaCO_3$. Pre-selected cells grown in 5 ml of MRS broth at 37°C for 18 hr were used for DNA sequencing.

The DNA was extracted using a commercial kit (Wizard® Genomic DNA Purification kit, Promega, Madison, WI, USA) with the following modifications. The samples were centrifuged (Mikro 200 R, Sigma-Aldrich, São Paulo, Brazil) at 10,000 *g* × 5 min and washed with saline solution (0.085 g/L). The cells were resuspended with 480 μl of EDTA (50 mM) and immediately added to 50 μl of lysozyme at 50 mg/ml. The concentration of extracted DNA was evaluated in a Nanodrop spectrophotometer (Thermo Scientific 2000, Waltham, MA, USA) and stocked at −20°C. The 16S rDNA gene sequence coding region was amplified by PCR in a PCR thermal cycler (Eppendorff®, Hamburg, Germany). The PCR products were determined directly with a sequencing kit using the prokaryotic 16S ribosomal DNA universal primers P027F (GAGAGTTTGATCCTGGC TCAG) and 1492R (TACGG(C/T)TACCTTGTTACGA CTT) (Heuer, Krsek, Baker, Smalla, & Wellington, 1997). The PCR reaction was performed in microfuge tubes containing 50 μl of the reaction mixture: DNA (approximately 60 ng); Reaction buffer 10X (Tris-HCl 0.1 mol/L, pH 8.0, KCl 0.5 mol/L); $MgCl_2$ (1.5 mmol/L, pH 8.0); dNTP mix (Promega, Madison, WI, USA); GoTaq® DNA Polymerase (Promega); Primer P027F (0.6 μmol/L), 1492R (0.6 μmol/L) and autoclaved milli-Q water. The reaction conditions used were as follows: initial denaturation (94°C × 5 min; 30 cycles); denaturation (94°C × 30 s; 60°C × 30 s); polymerization (72°C × 2 min); final extension (72°C × 5 min). The PCR reaction mixture was checked by run on agarose gel (14 g/L) electrophoresis with TRIS borate–EDTA buffer (Thermo Scientific). The gel was stained with 0.5 μg/ml ethidium bromide and bands were visualized on UV light. The PCR product was sent to the Macrogen© (Seoul, South Korea) for purification and sequencing. Sequence similarity searches were performed using the DNA database of GenBank, and the Basic Local Alignment Search Tool for nucleotide (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rDNA gene sequences that showed similarity >0.97 were

TABLE 1 Fermentation characteristics and epiphytic lactic acid bacteria succession of tropical maize silage

	Days of ensiling						
Items	**0**	**1**	**3**	**7**	**14**	**28**	**56**
Chemical composition (g/kg of dry matter)							
Dry matter (g/kg)	304	306	293	301	303	311	317
pH	6.05	3.79	3.80	3.69	3.60	3.66	3.7
WSC[a]	137	102	57.8	52.5	42.4	32.5	11.3
Lactic acid	-	13.5	20.1	27.5	30.8	41.0	35.0
Acetic acid	-	14.6	19.8	20.3	22.7	21.3	20.1
Propionic acid	-	1.19	1.29	1.52	1.49	1.70	1.13
Butyric acid	-	0.132	0.164	0.192	0.175	0.172	0.110
Microorganisms (log_{10} cfu[b] per g of fresh matter)							
Lactic acid bacteria	5.07	8.33	8.13	7.41	6.86	5.88	4.45
Yeasts and moulds	6.63	6.23	5.12	3.75	4.33	5.29	5.23
Enterobacteria	6.78	5.94	3.89	2.13	ND[c]	ND[c]	ND[c]
Epiphytic lactic-acid bacteria succession (log_{10} cfu[b] per g of fresh matter)							
Lactobacillus plantarum	1.98	3.50	5.85	6.30	5.56	3.94	-
Lactobacillus buchneri	-	-	-	-	-	-	2.67
Lactobacillus pentosus	0.304	-	0.650	0.370	1.30	0.235	-
Lactobacillus brevis	-	-	0.325	-	-	1.00	0.178
Lactobacillus casei	-	-	-	0.370	-	-	0.534
Lactobacillus fermentum	-	-	-	-	-	0.235	-
Lactobacillus sp.	-	-	-	-	-	0.235	-
Pediococcus pentosaceus	0.862	3.83	1.30	-	-	-	-
Weissella sp.	0.253	-	-	-	-	-	-
Weissella confuse	0.558	-	-	-	-	-	-
Weissella cibaria	1.11	1.00	-	-	-	-	-
Streptococcus salivarius	-	-	-	-	-	-	0.712
Uncultured	-	-	-	0.370	-	0.235	0.356

[a]Water-soluble carbohydrates.
[b]Colony-forming units.
[c]Not detected, < 2 log_{10} cfu/g of fresh matter.

considered belonging to the same Operational Taxonomic Unit (Altschul, Gish, Miller, Myers, & Lipman, 1990).

In another water-extract portion, the pH was measured using a potentiometer (Tecnal, SP, Brazil). After this, the water extract was filtered through Whatman 54 filter paper (Whatman, Florham, NJ), and 10 ml was acidified with 1:1 H_2SO_4 diluted with distilled water for the further chemical analysis (Table 1). The filtered and acidified water extracts were analysed for water-soluble carbohydrates (WSC) using glucose (Sigma-Aldrich, São Paulo, Brazil) to make the standard curve (Nelson, 1944). One millilitre of the acidified extract was centrifuged at 10,000 $g \times 15$ min, and subsequently analysed for lactic acid, acetic acid, propionic acid, butyric acid and ethanol by high-performance liquid chromatography (HPLC; SPD-10 AVP, Shimadzu, OR, USA) (Siegfried, Ruckemann, & Stumpf, 1984). The HPLC apparatus was equipped with a refractive index detector and used an Aminex HPX-87H column (BIO-RAD, CA, USA) with the mobile phase containing 0.005 M sulphuric acid, and a flow rate of 0.6 ml/min for organic acids and of 1.0 ml/min for ethanol, at 50°C.

2.3 | Characterization and screening of *Lactobacillus buchneri* strains

Among 151 LAB isolated, 15 were grouped with *L. buchneri* (Table 2). Physiological and biochemical tests were utilized for *L. buchneri* characterization and screening (Table 3 and Figure 1). All *L. buchneri* strains were cultivated in MRS broth for 16 hr at 37°C. After this period, the inoculum was standardized using a spectrophotometer (630 nm) at an optical density of 0.05, into 10 ml of MRS broth, which was incubated at 37°C with two replicates. The growth rate and the pH in MRS broth were evaluated each 3 hr. Growth rate was monitored by cell counting using drop plate and specific growth rate (per hr) was determined as the angular coefficient of the exponential growth line as a function of time ($LnX = \mu t + LnX_0$). Gas production in MRS broth was determined with a Durham tube at 37°C after 48 hr (Ávila et al., 2014). Growth at different temperatures (15, 37 and 45°C), pH (3.5, 4.5 and 8.5 at 37°C) and salt concentration (40 and 60 g/L of NaCl at 37°C) after incubation of 24 hr in MRS broth were

TABLE 2 *Lactobacillus buchneri* identification

Identification	Species	Similarity	GenBank access code
56.1	*Lactobacillus buchneri* FQ027	0.99	KF418820.1
56.2	*Lactobacillus buchneri* mze12	0.97	KP062948.1
56.4	*Lactobacillus buchneri* JNLAB-4	0.99	KC336485.1
56.6	*Lactobacillus buchneri* mze12	0.99	KP062948.1
56.7	*Lactobacillus buchneri* L4APL6.2	0.99	KM005146.1
56.8	*Lactobacillus buchneri* JNLAB-4	0.99	KC336485.1
56.9	*Lactobacillus buchneri* mze12	0.99	KP062948.1
56.21	*Lactobacillus buchneri* mze12	0.98	KP062948.1
56.22	*Lactobacillus buchneri* L4APL6.2	0.99	KM005146.1
56.24	*Lactobacillus buchneri* L4APL6.2	0.98	KM005146.1
56.25	*Lactobacillus buchneri* JNLAB-4	0.98	KC336485.1
56.26	*Lactobacillus buchneri* JNLAB-4	0.99	KC336485.1
56.27	*Lactobacillus buchneri* NRRL B-30929	0.98	CP002652.1
56.28	*Lactobacillus buchneri* FD2	0.99	JN188387.1
56.29	*Lactobacillus buchneri* MF161	0.98	KJ994455.1

Identification based on ~1,500 base pair gene that code for a portion of the 16S rDNA.

observed by absorbance in spectrophotometer (630 nm) (Santos et al., 2014). The growth in maize silage broth and fresh sugarcane broth was evaluated after 12 hr. Forage broth was crushed out from 100 g of herbage with 400 ml of distilled water in an industrial blender for 1 min, then filtered and sterilized (121°C, 15 min). Samples for metabolite production (lactic acid, acetic acid and ethanol) analyses using HPLC (same procedure as described before) were taken after 24 hr of growth in MRS broth at 37°C (Ávila et al., 2014) (Figure 1). Based on the characteristics of *L. buchneri* strains (Table 3 and Figure 1), eight strains were selected for evaluation as silage inoculant, and the selection criteria will be presented in the results section.

2.4 | Evaluation of *Lactobacillus buchneri* strains on silage fermentation

Two experiments were conducted, one with maize silage and other with sugarcane silage. The plants characteristics before ensiling are shown in Table 4. Eight *L. buchneri* strains isolated from maize silage were selected and evaluated, four in maize silage and four in sugarcane silage. Maize plants were harvested with the kernels at hard dough stage of maturity. Sugarcane (*Saccharum officinarum* L.) plants were harvested with approximately 16 months of growth. Whole plants were manually harvested and chopped at 2 cm theoretical length of cut using a JF-92 Z10 forage harvester (JF Agricultural Machinery, SP, Brazil). The isolated strains 56.22, 56.27, 56.28 and 56.29 were evaluated in maize silage. The strains 56.1, 56.4, 56.9, 56.26 and the commercial inoculant "Lalsil *Cana*" (*L. buchneri* NCIMB 40788; Lallemand, Goiás, Brazil) were evaluated in sugarcane silage. For all treatments, the theoretical application rate was 1.0×10^6 colony-forming units (cfu) per g of fresh weight, applied through 70 ml of cooled distilled water in 8 kg of chopped fresh forage. Maize and sugarcane silages without inoculants were used and applied just 70 ml of cooled-distilled water (control).

Inoculants were cultured in MRS broth for 16 hr, and then the inoculum was standardized using a spectrophotometer (630 nm) at an optical density of 0.05, into 20 ml of MRS broth and cultured for 12 hr. This schedule was obtained after the growth rate evaluation, which showed the maximum number of cells after incubation of 12 hr. With this, the amount of inoculum needed to reach 8.0×10^9 cfu/g was obtained. The amount of inoculum was centrifuged at 1,000 $g \times 10$ min and the supernatant discarded. Cells were resuspended with 70 ml distilled water and applied to achieve the final concentration of 1.0×10^6 cfu/g of fresh forage. Cells number was checked by cell counting using drop plate.

Chopped forage was mixed either with the inoculants or with cooled water (control) and approximately 7 kg of treated material was conditioned in plastic bucket (mini-silo; 25 cm diameter and 25 cm height, sealed with tight lids). Average packing density was 571 ± 16.7 kg/m^3 of fresh forage for maize silage and 573 ± 19.8 kg/m^3 for sugarcane silage. Four mini-silos (replicates) were prepared for each treatment. Mini-silos were stored at room temperature (25 ± 2°C) and opened after 90 days. Apparent DM loss was calculated using the weight and DM content of the fresh forage and silage (Jobim, Nussio, Reis, & Schmidt, 2007).

After mini-silo opening, samples of approximately 2 kg were removed from each mini-silo and returns to the plastic bucket to assess the aerobic stability. Temperatures were recorded every 30 min using data loggers (Escort mini; Impac, SP, Brazil) inserted into the silage mass at geometric centre using a ruler. Aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above the ambient temperature (25.6°C) according to Da Silva, Smith, Barnard, and Kung (2015). For fermentation characteristics, the forage samples were prepared as previously described.

2.5 | Statistical analyses

Data from the silo openings were analysed as a completely randomized design, with four replicates per treatment (inoculants). All microbial counts were converted into the logarithmic base ($\log_{10}$ cfu). Variance analysis and multiple comparisons of data were performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA) and the means were separated by Tukey's test ($p \leq .05$).

TABLE 3 Phenotypic characteristics of *Lactobacillus buchneri* strains isolated from tropical maize silage after incubation for 24 hr

Items	56.1	56.2	56.4	56.6	56.7	56.8	56.9	56.21	56.22	56.24	56.25	56.26	56.27	56.28	56.29
Growth at pH															
3.5	+	+	+	++	+	++	++	+	+	+	+	++	++	+	+
4.5	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	++++
8.5	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Growth at temperature (°C)															
15	+	+	+	+++	+	++++	+++	+	+	+	+	+++	++	+++	+
37	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
45	++	+++	+++	++++	+++	++++	+++	+++	+++	++	++	+++	+++	+++	++
Growth in NaCl (g/L)															
40	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++
60	+	+++	++	+++	+	+++	+++	+++	++++	+++	+	+++	++	+++	+
Carbon dioxide[a]	++	+++	+++	+	+++	+	+	+++	+++	+++	+++	+	+++	+	+++
pH[b]	4.45	4.38	4.45	3.86	4.42	4.44	4.46	4.44	4.53	4.44	4.45	3.91	4.45	3.86	4.43
μ[c] (per hr)	0.136	0.097	0.167	0.161	0.182	0.239	0.197	0.193	0.202	0.158	0.162	0.314	0.205	0.194	0.203
Cells count at broth[d] (log_{10} cfu/ml)															
Maize silage	7.76	7.90	7.71	7.74	6.48	7.08	7.57	7.45	6.96	6.87	7.38	7.23	6.65	6.52	6.74
Fresh sugarcane	8.02	8.08	8.04	8.16	7.43	7.45	7.57	7.60	7.34	7.43	7.40	8.53	7.67	8.08	7.58
MRS	8.72	8.85	8.72	9.32	9.30	9.52	9.83	9.38	9.60	9.45	9.49	9.38	9.32	9.34	9.32

Growth measured by optical density (630 nm): <0.3 = +; >0.3 to <0.6 = ++; >0.6 to <0.9 = +++; >0.9 = ++++.
[a]Gas in MRS broth with Durham tubes: <1/3 of tube = +; >1/3 to <2/3 = ++; >2/3 = +++.
[b]pH in MRS broth with initial pH 6.
[c]μ = specific growth rate.
[d]Forage broth = 100 g of forage mixed with 400 ml of distilled water; MRS = de Man, Rogosa and Sharpe broth.

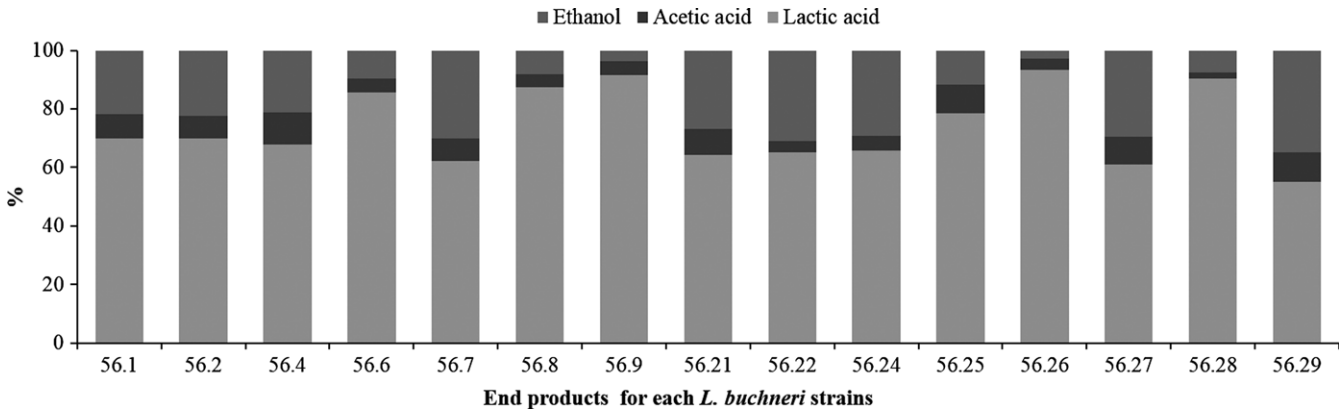

FIGURE 1 Lactic acid, acetic acid and ethanol production by *Lactobacillus buchneri* strains after 24 hr in MRS broth

3 | RESULTS

3.1 | Isolation and identification of LAB from tropical maize silage

Maize silage fermentation and identification of isolated LAB are shown in Table 1. The DM average was 305 g/kg. The fermentation pattern was typical from whole-plant maize silage. The pH dropped quickly in the first days of fermentation. In all, 151 strains of LAB were identified based on sequence analyses of their 16S rDNA. All of then showed at least 97% of similarity and were categorized as belonging to the same genetic group.

In the first three days of ensiling, the LAB groups *Pediococcus pentosaceus*, *Lactobacillus plantarum* and *Weissella* sp. were the most representative. *L. plantarum* was the predominant group until the 28th day of ensiling. At day 56 of ensiling, the predominant groups were *L. buchneri* followed by *Streptococcus salivarius* and *L. casei*. The predominant groups of tropical maize silage, considering all isolates in MRS agar were as follows: *Lactobacillus plantarum* 53.0%, *Pediococcus pentosaceus* 11.9%, *L. buchneri* 9.9%, *L. pentosus* 5.3%, *Weissella cibaria* 4.6% and

TABLE 4 Plant characterization before ensiling

Item	Maize	Sugarcane
Dry matter (g/kg)	355	319
Water-soluble carbohydrates (g/kg of DM[a])	94.1	385
pH	5.76	5.54
Lactic acid bacteria (log_{10} cfu[b] per g of FM[c])	6.87	6.29
Yeasts and moulds (log_{10} cfu[b] per g of FM[c])	5.86	6.63

[a]Dry matter.
[b]Colony-forming units.
[c]Fresh matter.

L. brevis 4.0%. The similarity of 16S rDNA sequence compared to the access code in GenBank for the *L. buchneri* strains is shown in Table 2.

3.2 | Screening of *Lactobacillus buchneri* strains based on phenotypic characteristics and metabolite production

Strains with the same access code in GenBank showed differences on phenotypic characteristics (Table 3). Most of the strains decreased the growth when cultured in pH 3.5 or 15°C. All strains showed the highest growth with 40 g/L of NaCl. In MRS broth, all strains produce lactic acid, acetic acid and ethanol (Figure 1).

The strains 56.22 (highest growth rate), 56.27 (great growth at acid pH), 56.28 (highest lactic acid production and lower final pH) and 56.29 (high acetic acid production) were selected to be inoculate in maize silages. For sugarcane silage, the strains 56.1 (high acetic acid production), 56.4 (highest acetic acid production), 56.9 (great growth at high temperature) and 56.26 (lowest ethanol production) were selected. Different *L. buchneri* strains were used in each crop to evaluate a larger number of strains.

3.3 | Effects of *Lactobacillus buchneri* strains on the fermentation characteristics and aerobic stability of maize silage

Selected strains did not affect the DM content, yeast and moulds population, DM loss, WSC, lactic acid, propionic acid, butyric acid and ethanol of maize silage after 90 days of ensiling ($p > .050$). The pH, LAB population and concentration of acetic acid were affected by inoculants ($p < .050$). The strains 56.27, 56.28 and 56.29 showed lower pH than the untreated control silage. The LAB population was greater for the silages treated with the strains 56.22 and 56.27 in comparison with the untreated control silage ($p = .003$). The strains 56.22, 56.28 and 56.29 decreased the concentration of acetic acid in comparison with the untreated control silage ($p = .041$; Table 5).

After 7 days of air exposure, there was no effect of inoculation with the *L. buchneri* strains on the aerobic stability of silages ($p = .289$). Silages inoculated with the strains 56.27, 56.28 and 56.29 increased the population of yeasts and moulds compared to the control after air exposure ($p = .001$). Overall mean for aerobic stability of maize silage was of 32.7 hr. Both treated and untreated silages showed high pH, high number of yeasts and moulds and low concentrations of WSC and organic acids after air exposure (Table 5).

3.4 | Effects of *Lactobacillus buchneri* strains on fermentation characteristics and aerobic stability of sugarcane silage

The treatment with *L. buchneri* affected the DM content, LAB population, yeasts and moulds population, DM losses, and concentrations of WSC, lactic acid and ethanol of sugarcane silage after 90 days of ensiling ($p < .050$; Table 6). Silage pH and the concentrations of acetic, propionic and butyric acids were not affected by the treatments ($p > .050$). The silages inoculated with the strains 56.1, 56.4 and 40788 showed the highest DM content ($p = .004$). Higher LAB population compared with the untreated control silage was observed in the silages treated with the strains 56.4 and 56.9 ($p = .015$). Inoculation with the strains 56.1, 56.9 and 40788 decreased the population of yeasts and moulds in comparison with untreated control silage ($p = .024$). Dry-matter loss decreased related to untreated control silage when the silages were treated with the strains 56.1, 56.4 and 40788 ($p < .001$). The highest concentration of WSC was observed for the silages inoculated with the strains 56.1 and 56.4 ($p < .001$). Compared with the untreated control silage, the strains 56.1, 56.4 and 40788 showed lower concentration of lactic acid ($p = .023$). Regarding the ethanol concentration, silages inoculated with 56.1, 56.4 and 40788 showed lower values than the inoculated silages with the strains 56.9 and 56.26, whereas the untreated control silage showed intermediate values ($p = .016$).

After 7 days of air exposure, there was no effect of the inoculation with *L. buchneri* on the aerobic stability, maximum temperature reached, DM content, pH, concentrations of WSC, lactic acid, butyric acid and ethanol in sugarcane silage ($p > .050$; Table 6). Overall mean for aerobic stability was of 58.2 hr ($p = .524$). The inoculated silage with the strain 56.9 showed higher population of yeasts and moulds than the untreated control silage; other strains showed intermediates values ($p = .024$). The strains 56.1, 56.4 and 40788 showed lower acetic acid and propionic acid concentrations than the untreated control silage after air exposure ($p = .001$).

4 | DISCUSSION

4.1 | Isolation and identification of LAB from tropical maize silage

Whole-crop maize had appropriate characteristics to produce high-quality silage. Results of our study demonstrated that there is a quick drop in the pH with the consumption of WSC and production of organic acids, besides the epiphytic LAB succession during the pre-ensiling and ensiling periods. This fermentation pattern occurs due to the adequate contents of DM, WSC and buffering capacity.

Isolation of LAB using MRS medium under anaerobic conditions, followed by the analyses of 16S rDNA fragment, allows the

TABLE 5 Fermentation pattern and aerobic stability (g/kg of DM, unless stated otherwise) of maize silage treated with *Lactobacillus buchneri* strains after 90 days of ensiling and after 7 days of air exposure at 23°C (*SEM*, standard error of mean)

		L. buchneri strains					
Items	**Control**	**56.22**	**56.27**	**56.28**	**56.29**	***SEM***	***p* value**
After 90 days of ensiling							
DM[†]	368	378	378	365	377	1.58	.315
pH	3.77[a]	3.74[ab]	3.72[b]	3.73[b]	3.72[b]	0.005	.015
LAB[‡]	4.35[b]	6.19[a]	6.66[a]	5.45[ab]	5.28[ab]	0.216	.003
Y&M[§]	5.39	5.80	5.65	5.03	5.12	0.181	.633
DM loss	30.7	30.1	33.2	33.2	32.6	0.540	.213
WSC[¶]	1.51	1.39	1.65	1.27	1.30	0.066	.348
Lactic acid	38.8	34.5	40.9	39.7	40.1	1.16	.446
Acetic acid	12.2[a]	9.81[b]	11.0[ab]	9.92[b]	9.64[b]	0.310	.041
Propionic acid	0.552	0.574	0.495	0.443	0.692	0.035	.129
Butyric acid	6.58	3.90	3.96	2.97	2.96	0.471	.115
Ethanol	11.4	15.7	19.8	18.2	16.0	1.19	.282
After 7 days of air exposure at 23°C							
Stability (hours)	31.9	32.5	32.9	28.9	37.4	1.24	.289
Max. temp (°C)	39.0	38.9	38.5	38.8	37.5	0.250	.328
DM[†]	338	345	348	338	346	2.36	.590
pH	6.11	6.18	6.24	6.30	6.22	0.029	.395
Y&M[§]	7.93[c]	8.36[bc]	8.77[ab]	8.99[a]	8.96[a]	0.098	.001
WSC[¶]	2.91	4.02	5.00	3.94	6.47	0.452	.129
Lactic acid	7.09	4.52	5.40	5.68	4.66	0.331	.117
Acetic acid	1.91	2.26	2.40	1.90	1.96	0.133	.691
Propionic acid	0.332	0.385	0.462	0.263	0.281	0.033	.195
Butyric acid	1.56	2.16	1.58	2.21	3.57	0.275	.109
Ethanol	2.35[b]	5.82[ab]	3.59[b]	15.8[a]	14.8[a]	1.732	.014

[†]Dry-matter content (g/kg of fresh matter).
[‡]Enumeration of lactic acid bacteria (log_{10} cfu/g of fresh matter).
[§]Enumeration of yeasts and moulds (log_{10} cfu/g of fresh matter).
[¶]Water-soluble carbohydrates (g/kg of dry matter).
[a–c]Means in rows with unlike superscripts differ ($p < .05$).

identification of different species in fresh forage and silage (Ávila et al., 2014; Ni et al., 2015). In our study, the growth of *L. plantarum*, *P. pentosaceus* and *W. cibaria* was replaced by *L. buchneri*, *S. salivarius*, *L. casei* and *L. brevis* during the silage fermentation. Substrates availability and pH could be factors that induce the shift of LAB species. Our observations that the *L. buchneri* accounted for a large proportion at day 56 of ensiling can be due their ability to utilize lactic acid as energy source in acidic pH (Oude Elferink et al., 2001).

Several studies have investigated the diversity of epiphytic LAB during the maize ensiling process. In general, *L. plantarum* and *P. pentosaceus* were the predominant LAB species in the beginning of fermentation, whereas *L. buchneri* and *L. brevis* were widely detected few days after ensiling (Brusetti et al., 2006; Lin, Bolsen, Brent, & Fung, 1992; Parvin, Wang, Li, & Nishino, 2010; Stevenson, Muck, Shinners, & Weimer, 2006; Zhou et al., 2016). It is generally recognized that to produce a high-quality silage, the fermentation should start with homofermentative LAB species. These species quickly grow and decrease the pH. However, when the pH becomes acid and substrate limited, heterofermentative LAB species can replace them and eventually predominate (McDonald, Henderson, & Heron, 1991). This is important in maize silage because the acetic acid produced by the heterofermentative LAB can increase the aerobic stability by controlling the yeasts growth, and this effect is more consistent after 50 days of ensiling (Kleinschmit & Kung, 2006b).

In our study, the identification of microorganisms at the species level may be considered incomplete because some closely related species are difficult or impossible to distinguish based on their genotypes (Hammes & Hertel, 2006). Doi et al. (2013) analysed the nucleotide sequences of the V2-V3 region of 16S rRNA, 16S-23S rRNA intergenic spacer regions, RAPD-PCR, DNA–DNA hybridization, G+C content and immuno-identification of silage microorganisms. According to these authors, it was still not possible to

TABLE 6 Fermentation pattern and aerobic stability (g/kg of DM, unless stated otherwise) of sugarcane silage treated with isolated *Lactobacillus buchneri* strains after 90 days of ensiling and after 7 days of air exposure at 23°C (*SEM*, standard error of mean)

		L. buchneri strains						
Items	**Control**	**56.1**	**56.4**	**56.9**	**56.26**	**40788**	***SEM***	***p* value**
After 90 days of ensiling								
DM†	312^{c}	346^{a}	347^{a}	340^{ab}	335^{bc}	345^{a}	5.71	.004
pH	3.39	3.42	3.44	3.48	3.47	3.50	0.014	.192
LAB‡	6.35^{c}	6.72^{bc}	7.46^{ab}	7.77^{a}	6.60^{c}	6.69^{bc}	0.146	.015
Y&M§	5.56^{a}	4.79^{bc}	4.93^{abc}	4.66^{bc}	5.19^{ab}	4.42^{c}	0.110	.024
DM loss	260^{ab}	137^{c}	146^{c}	209^{bc}	307^{a}	151^{c}	16.0	<.001
WSC¶	22.2^{c}	73.6^{a}	70.1^{a}	49.3^{b}	26.6^{c}	35.0^{bc}	4.78	<.001
Lactic acid	59.8^{a}	36.5^{b}	38.0^{b}	45.9^{ab}	57.9^{a}	38.9^{b}	2.82	.023
Acetic acid	33.0	38.0	37.1	35.5	25.7	36.0	2.31	.716
Propionic acid	3.66	3.33	3.61	3.16	2.73	3.44	0.143	.447
Butyric acid	3.35	4.01	4.19	3.82	3.50	2.95	0.215	.628
Ethanol	216^{ab}	141^{b}	154^{b}	318^{a}	385^{a}	152^{b}	21.1	.016
After 7 days of air exposure at 23°C								
Stability (hours)	56.0	54.5	60.5	50.6	67.2	60.6	2.51	.524
Max. temp. (°C)	39.5	38.0	43.0	41.5	38.0	37.4	0.780	.221
DM†	262	272	272	280	251	282	4.90	.526
pH	3.49	4.13	4.36	3.59	3.63	4.91	0.170	.078
Y&M§	7.28^{b}	9.10^{ab}	9.41^{a}	8.78^{ab}	7.90^{ab}	8.72^{ab}	0.230	.024
WSC¶	34.0	41.3	24.7	55.2	27.7	45.0	4.29	.327
Lactic acid	19.5	10.6	8.11	14.3	12.9	12.4	1.16	.079
Acetic acid	144^{a}	23.6^{bc}	6.46^{c}	93.4^{ab}	131^{a}	29.7^{bcd}	12.9	<.001
Propionic acid	15.2^{a}	7.01^{bcd}	4.10^{d}	10.0^{abcd}	14.1^{ab}	5.97 ^{cd}	1.06	.001
Butyric acid	2.51	1.41	1.88	3.55	2.04	2.59	0.261	.251
Ethanol	46.0	45.2	79.4	31.1	75.7	31.0	13.4	.868

†Dry-matter content (g/kg of fresh matter).
‡Enumeration of lactic acid bacteria (log_{10} cfu/g of fresh matter).
§Enumeration of yeasts and moulds (log_{10} cfu/g of fresh matter).
¶Water-soluble carbohydrates (g/kg of dry matter).
$^{a–c}$Means in rows with unlike superscripts differ ($p < .05$).

distinguish some phylogenetically closely related species, such as *L. plantarum, L. paraplantarum and L. pentosus*; *L. casei*, *L. paracasei* and *L. rhanmosus*; and *L. buchneri*, *L. kefiri*, *L. parabuchneri* and *L. rapi*.

4.2 | Screening of *Lactobacillus buchneri* strains

Lactobacillus buchneri strains were screened in an attempt to identify the strains with potential for silage inoculant because the strains of same species might show differences in the metabolism and in the ability to survive in the silage environment (Santos et al., 2015). In addition, different strains of the same species generally result in distinct patterns of silage fermentation because their physiology and metabolism can be different, which makes difficult the screening process (Ávila et al., 2014; Carvalho et al., 2014; Saarisalo, Skytta, Haikara, Jalava, & Jaakkola, 2007).

In our study, the *L. buchneri* strains screened to be used as inoculants in silages were based mainly on growth rate and metabolites production to improve the fermentation and/or aerobic stability. Effects of microbial inoculants on silage fermentation are mainly due to the ability of quickly dominating the fermentation and produce metabolites, besides the changes in the pH. In addition, the crop and the LAB selected must be compatible to achieve the aim of inoculation. Thus, those characteristics can be employed as a selection criterion of new inoculants (Ávila et al., 2014; Ni et al., 2015; Saarisalo et al., 2007).

4.3 | Effects of *Lactobacillus buchneri* strains on maize silage

In our study, the DM and WSC contents of fresh chopped maize (355 and 94.1 g/kg, respectively; Table 4) at ensiling were considered adequate for ensiling process (McDonald et al., 1991). All silages showed good fermentation pattern and low DM loss, which is expected in maize silage due to its adequate concentrations of

DM and WSC in the fresh crop (Table 4). The average values of pH observed in the present study for all silages range in the pH interval considered adequate for the maize silage (Kung & Shaver, 2001). However, the inoculated silages, except the one treated with the strain 56.27, showed a slight decrease in the pH when compared to the untreated control silage. It was not expected because some *L. buchneri* strains can degrade lactic acid into acetic acid raising the pH (Driehuis et al., 1999; Kung, Stokes, & Lin, 2003; Oude Elferink et al., 2001). However, that difference can be considered quite low to be discussed biologically.

Although none improvement of inoculant was observed on silage fermentation, there is evidence that the strains survival due to the great LAB count is observed in the inoculated silages. The greater LAB population in the inoculated silages compared to the untreated control silage probably was due to the fact that in acid conditions some LAB may decrease viability, and just specialized LAB, such as *L. buchneri*, can remain active (Oude Elferink, Driehuis, Gottschal, & Spoelstra, 2000). Reduction of LAB population during the fermentation process also has been reported by other authors; it is related to resistance of LAB to acidic conditions (Assis, Ávila, Pinto, & Schwan, 2014; Li & Nishino, 2011).

In our study, populations of yeasts and moulds in the fresh forage were greater than the previous studies with maize silage (Assis et al., 2014; Filya, 2003; Zhou et al., 2016). This difference in population could be attributed to factors such as maize hybrid, maturity and environmental conditions. However, this high population also can be a reason because the treatments had no effect on population of yeasts and moulds. However, the ethanol contents of silages in this study are in the interval acceptable (Kung & Shaver, 2001; Li & Nishino, 2011).

After air exposure, all silages spoiled and had high population of yeast and moulds. The high DM content and low concentration of acetic, propionic and butyric acids, and ethanol of silages can be due to the volatilization. In addition, yeasts and moulds, and aerobic bacteria can metabolize WSC and lactic acid under aerobic conditions. Furthermore, because of the reduction in the concentrations of lactic, acetic, propionic and butyric acids, the pH increased (Carvalho et al., 2014; McDonald et al., 1991; Oude Elferink et al., 2001).

Screening of *L. buchneri* strains based on growth at high temperature and acid pH, and acetic acid production had no improvement on the fermentation and aerobic stability of maize silage. In our study, the lactic acid was proportionally the major product of the fermentation for all *L. buchneri* strains. The great population of yeasts and moulds in the fresh crop and the absence of metabolites produced by the *L. buchneri* strains in sufficient amounts, as acetic acid that can inhibit these microorganisms (Kung et al., 2003), can explain the results of aerobic stability. Since the inoculants were isolated from maize crop, probably they exist quite frequently also in the untreated control maize silage. Thus, this also can be a reason for the absence of improvements in maize silage.

Based on our study and others, we suggest that the effect of isolated strains on the aerobic stability of maize silages can be difficult to be predicted based on the metabolic profile and growth rate, which suggests that the way to find an heterofermentative LAB is beyond the criteria postulated for an inoculant with homofermentative LAB (McDonald et al., 1991; Woolford, 1984).

4.4 | Effects of *Lactobacillus buchneri* strains on sugarcane silage

In our study, the high DM content and low DM losses in the inoculated silages with the strains 56.1, 56.4 and 40788 are mainly due to higher content of residual WSC. Acetic acid concentration in these silages was not different compared with the untreated control silage, and the inoculant effect on the population of yeasts and moulds was variable. This variation is probably due to the inhibitory effect of ethanol on yeasts population of sugarcane silage (Ávila et al., 2014; Carvalho et al., 2014).

Inoculated silages with the strains 56.1, 56.9 and 40788 showed significantly decrease in the population of yeasts and moulds in comparison with the untreated control silage. Inoculated silages with the strains 56.1, 56.4 and 40788 showed better-quality fermentation than the untreated control silage because of the preservation of WSC and decreased concentration of ethanol. Despite the absence of changes in the acetic acid concentration by itself, we highlight that the concentration of lactic acid was lower than the untreated control in those silages, which would increase the proportion of acetic acid among the acids produced in the silo. Indeed, those isolated strains were selected according to the highest production of acetic acid, as noted in the study of Ávila et al. (2014).

Inoculants containing heterofermentative LAB that produces high concentrations of acetic acid are more suitable for yeast control because of the inhibitory effect of this acid (Kung et al., 2003). Some *L. buchneri* strains do not have the ability to reduce acetylphosphate to ethanol, possibly due to lack of acetaldehyde dehydrogenase, and thus increase the concentration of acetic acid as a final product of the fermentation (Axelsson, 2004). The fungicidal effect of acetic acid is due to lipophilicity. In acid pH, the acetic acid can permeate the cell membrane; inside the cell, in neutral pH, the disassociation of acetic acid releasing protons, which decreases the intracellular pH and can lead the microorganisms to death (Danner, Holzer, Mayrhuber, & Braun, 2003).

In sugarcane silage, the increase in ethanol concentration is normally associated with fermentation of WSC and organic acids by yeast. The effect of experimental inoculants was more expressive in sugarcane silages in comparison with maize silages because of the high concentration of WSC in fresh sugarcane and the intensity of the fermentation process in this crop. The results also suggest that a strain selected from maize silage was more effective in sugarcane silage because of their absence in the fresh crop. In addition, some heterofermentative LAB, such as *L. buchneri*, can convert sugars into ethanol (Liu, Skinner-Nemec, & Leathers, 2008). This could explain the increased ethanol concentration of inoculated silages with the strains 56.9 and 56.26.

The population of yeasts and moulds in the fresh sugarcane was higher than in other published studies (Ávila et al., 2014). According to McDonald et al. (1991), silages containing population of yeast and moulds larger than five-log cfu/g are more susceptible to aerobic deterioration. In addition, the high concentration of lactic acid and

residual WSC of good quality silage are substrate for yeast, moulds and aerobic bacteria.

In general, the *L. buchneri* can enhance the fermentation of sugarcane silage resulting in low DM loss and aerobic stability increased (Ávila, Pinto, Figueiredo, & Schwan, 2009; Ávila et al., 2014; Pedroso et al., 2008; Roth et al., 2010). However, the improvement on aerobic stability can be due to other antimicrobial substance, besides the acetic acid. For example, some *L. buchneri* strains can produce bacteriocin that may be responsible to enhance the aerobic stability (Kleinschmit & Kung, 2006a; Yildirim, 2001).

In our study, after air exposure, all silages spoiled and had high population of yeasts and moulds. Beyond the effects described for the maize silage experiment, the sugarcane silages that showed a high concentration of ethanol resulted in high concentration of acetic acid and low pH after air exposure. This pattern can be due to the effects of aerobic acetic bacteria; belong to family *Pseudomonodaceae*, mainly *Acetobacter* and *Gluconobacter* genera; this bacteria can convert WSC and/or ethanol into acetic acid, aerobically (Rizzon, 2006).

5 | CONCLUSION

Lactobacillus buchneri predominated at day 56 of ensiling maize. Screening of *L. buchneri* strains based mainly on acetic acid production showed the best results for sugarcane silage. *L. buchneri* 56.1 and 56.4 are considered the most suitable strains for improving the fermentation of sugarcane silage and thus are potential inoculants for silage production. However, authors recommend that the strains have to be tested in a farm-scale silo to check their real effects.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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