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RESEARCH ARTICLE

Effect of Wild *Lactobacillus buchneri* Strains on the Fermentation Profile and Microbial Populations of Sugarcane Silage

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Abstract: ***Background*:** Sugarcane silage has been increasing as a feed in the tropics by dairy farmers. However, sugarcane 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. There are several patents that have recently shown the benefits of applying *Lactobacillus buchneri* in forage preservation.

***Objective*:** This study aimed to investigate the changes in pH, DM, water-soluble carbohydrates (WSC) and fermentation end product concentrations that occur in sugarcane silage with or without inoculation with *L. buchneri* after 45 days of ensiling.

***Method*:** Sugarcane plants were harvested with approximately 16 months of growth and chopped at 2 cm. Four strains of wild *L. buchneri* (56.1, 56.4, 56.9 and 56.26) and the commercial inoculant "Lalsil Cana" were evaluated. For all treatments, the theoretical application rate was 1.0×10^6 colony-forming units (cfu) per g of fresh weight. Data from the silo openings were analysed as a completely randomized design, with four replicates per treatment (inoculants).

***Results*:** The treatment with *L. buchneri* affected the DM content, pH, lactic acid bacteria (LAB) population, DM recovery, and concentrations of WSC, lactic acid, acetic acid and ethanol of sugarcane silage after 45 days of ensiling. Yeasts and molds populations and the concentrations of propionic and butyric acids were not affected by the treatments.

***Conclusion*:** *Lactobacillus 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. At present, we are preparing the patent application.

ARTICLE HISTORY

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1. INTRODUCTION

Grazing is the most common and economical way to feed cattle, however, it cannot be done over the entire year, due to the climatic conditions that limit the grasses growth. In the winter, for example, there is no forage production enough to feed the animals [1]. The choice of suitable forage conservation process to constantly provide feed, essentially depends on the climatic conditions at harvest. In hot areas with dry seasons, probably haymaking is the best choice for forage preservation, because it is a simple technology. However, in tropical regions with hot and humid climates, it is difficult to produce high-quality hay, due to high humidity and frequent rainfall at optimum stage of maturity for a crop with better nutritional value. In this context, ensiling is an important method of forage preservation because it is not too dependent on weather as haymaking. In addition, in many parts of world, the silage is the major source of energy in the total mixed rations of ruminants [2]. In addition, properly made and managed silage is an excellent feed that poses no health risks to humans or livestock.

Sugarcane (*Saccharum officinarum* L.) silage has been increasing as a feed in the tropics by dairy farmers [3], mainly because of its high yield and low production cost. However, sugarcane has high sugar content and low buffering capacity, which favour lactic acid production and fast pH drop, but normally has high yeast population that leads to intense alcoholic fermentation and excessive dry-matter (DM) loss during ensilage and after air exposure, as well [4].

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Biological silage additives can assist in making well-preserved silages by promoting a rapid reduction in silage pH and preventing aerobic deterioration [5]. There are several patents that have recently shown the benefits of applying *L. buchneri* in forage preservation [6]. *Lactobacillus buchneri* application in silages can reduce DM losses and increase the aerobic stability, degradability rate and animal performance [7, 8]. This obligate heterolactic acid bacterium increases acetic acid concentration and decreases yeast and mold of silage; however, the effects are strain-specific and dose-dependent [9].

In addition, the *L. buchneri* inoculants may be more cost effective than chemicals additives. However, the current inoculants require a minimum of 45 to 60 d storage before substantial benefits [10]. Greater concentrations of acetic acid, a hallmark of silages treated with *L. buchneri*, were observed from 56 d of ensiling onward [11], making them a poor choice in those circumstances where silage is fed after a short storage period [10]. Identifying strains that would improve the acetic acid concentration and aerobic stability earlier in the ensiling process would be helpful [9].

This study aimed to investigate the changes in pH, DM, water-soluble carbohydrates (WSC) and fermentation end product concentrations that occur in sugarcane silage with or without inoculation with *L. buchneri* after 45 days of ensiling.

2. MATERIALS AND METHODS

2.1. Silage Preparation

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 plants characteristics before ensiling are shown in Table **1**.

Table 1. Plant characterization before ensiling.

	Fresh Sugarcane
Dry matter (% of fresh matter)	31.9
Water-soluble carbohydrates (% of dry matter)	38.5
pH	5.54
Lactic acid bacteria (log cfu/g of fresh matter)	6.29
Yeast and mold (log cfu/g of fresh matter)	6.63

The wild *L. buchneri* strains isolated from tropical maize silage were identified according to [12]. For the inoculants preparation, these strains were cultured in de Man, Rogosa and Sharpe (MRS) broth for 16 h, 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 h. This schedule was obtained after growth rate evaluation, which showed the maximum number of cells after incubation of 12 h. With this, the amount of inoculum needed to reach 8.0×10^9 colony-forming units (cfu) per g was obtained. The amount of inoculum was centrifuged at 1,000 g × 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 in 8 kg of fresh forage. After application, cells number was checked by cell counting using drop plate.

The treatments were four wild strains of *L. buchneri* (56.1, 56.4, 56.9 and 56.26) and the commercial inoculant Lalsil Cana® (*L. buchneri* strain NCIMB 40788, Lallemand, Goiás, Brazil). For all treatments, the theoretical application rate was 1.0×10^6 cfu/g of fresh weight, applied through 70 ml of cooled distilled water in 8 kg of chopped fresh forage. Sugarcane silage without inoculant was used and applied just 70 ml of cooled-distilled water (control). Chopped forage was mixed either with the inoculants or just cooled water (control) and approximately 500 g of treated material was conditioned in nylon-polyethylene bags and vacuum sealed (25 × 35 cm; Doug Care Equipment Inc., Springville, CA; Eco vacuum 1040, Orved, Italy). Four mini-silos (replicates) were prepared for each treatment. Mini-silos were stored at room temperature (25 ± 2°C).

2.2. Laboratory Analysis

After 45 d of ensiling, the mini-silos were opened for analyzing fermentation quality. Dry matter (DM) was analyzed according to AOAC Methods 934.01 [13]. Wet silage (25 g) was homogenized with 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. Pour plates were prepared with MRS agar (Difco, São Paulo, Brazil) for LAB, and Potato Dextrose Agar (PDA; Difco, Sao Paulo, Brazil) containing 1.5% of tartaric acid solution (10% w./v.) for yeast and mold. The MRS plates were incubated at 37°C for 48 h in the anaerobic jars (Permution, Curitiba, PR, Brazil). The PDA plates were incubated aerobically at 25°C for 5 d. All colonies were counted on plates with 25–250 well-isolated colony-forming units.

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 further chemical analysis. The filtered and acidified water extracts were analysed for WSC using glucose (Sigma-Aldrich, São Paulo, Brazil) to make the standard curve [14]. One millilitre of the acidified extract was centrifuged at 10,000 g × 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) [15]. 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.

Apparent DM loss was calculated using the weight and DM content of the fresh forage and silage [16]. The DM content was corrected for volatile compounds [17].

Table 2. The dry matter (DM) content, pH, number of lactic acid bacteria (log cfu/g of FM), number of yeasts and molds (log cfu/g of FM), and DM recovery of sugarcane silage treated with isolated *Lactobacillus buchneri* strains after 45 d of ensiling.

-	Control	*L. buchneri* Strains					SEM[1]	P-value
		56.1	56.4	56.9	56.28	NCIMB 40788		
Dry matter (% of FM[3])	24.5^{bc}	30.1^{ab}	30.9^{a}	24.9^{bc}	23.2^{c}	30.4^{ab}	0.489	<0.001
pH	3.56^{ab}	3.47^{b}	3.49^{b}	3.61^{a}	3.67^{a}	3.53^{ab}	0.015	0.006
Lactic acid bacteria	7.40^{b}	8.36^{a}	8.47^{a}	8.35^{a}	7.40^{b}	8.48^{a}	0.138	0.001
Yeasts and molds	3.66	5.08	4.96	3.64	4.59	4.19	0.115	0.059
DM recovery (%)	78.5^{b}	92.1^{a}	93.2^{a}	75.9^{b}	71.9^{b}	92.0^{a}	1.45	<0.001

[1]Standard error of mean. [2] Fresh matter. [a–c]Means with different letters within a row differ ($p< 0.05$).

Table 3. The chemical composition (% of dry matter) of sugarcane silage treated with isolated *Lactobacillus buchneri* strains after 45 d of ensiling.

-	Control	*L. buchneri* Strains					SEM[1]	P-value
		56.1	56.4	56.9	56.26	NCIMB 40788		
WSC[2]	1.51^{d}	8.06^{ab}	9.80^{a}	2.53^{cd}	2.12^{cd}	5.85^{bc}	0.438	<0.001
Lactic acid	4.08^{ab}	3.22^{b}	3.99^{ab}	4.79^{a}	4.44^{ab}	4.69^{a}	0.183	0.024
Acetic acid	1.25^{c}	2.74^{ab}	3.37^{ab}	2.68^{b}	1.17^{c}	4.06^{a}	0.187	0.005
Propionic acid	0.187	0.196	0.304	0.241	0.279	0.204	0.012	0.358
Butyric acid	0.316	0.293	0.34	0.43	0.365	0.327	0.016	0.664
Ethanol	17.8^{ab}	4.77^{b}	3.86^{b}	10.4^{ab}	23.5^{a}	5.45^{b}	2.22	0.008

[1]Standard error of mean. [2]Water-soluble carbohydrates. [a–c]Means with different letters within a row differ ($p<0.05$).

2.3. Statistical Analysis

Data from the silages 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 ($p\leq0.05$).

3. RESULTS

The treatment with *L. buchneri* affected the DM content, pH, LAB population, DM recovery, and concentrations of WSC, lactic acid, acetic acid and ethanol of sugarcane silage after 45 days of ensiling ($p<0.05$). Yeast and mold population and the concentrations of propionic and butyric acids were not affected by the treatments ($p>0.05$).

The silages inoculated with the strains 56.1, 56.4 and the commercial strain NCIMB 40788 showed the highest DM content ($p<0.001$). Higher LAB population compared with the untreated control silage was observed in the silages treated with the strains 56.1, 56.4 and 56.9 ($p=0.001$). Dry-matter recovery increased related to untreated control silage when the silages were treated with the strains 56.1, 56.4 and NCIMB 40788 ($p<0.001$; Table **2**).

The highest concentration of WSC was observed for the silages inoculated with the strains 56.1 and 56.4 ($p<0.001$). Compared with the untreated control silage, the strains 56.1, 56.4, 56.9 and NCIMB 40788 showed greater concentration of acetic acid ($p=0.005$). Regarding the ethanol concentration, silages inoculated with 56.1, 56.4 and NCIMB 40788 showed lower values than the inoculated silages with the strain 56.26, whereas the untreated control silage showed intermediate values ($p=0.008$; Table **3**).

4. DISCUSSION

In our study, the high DM content and DM recovery in the inoculated silages with the strains 56.1, 56.4 and NCIMB 40788 are mainly related to a reduction in ethanol concentration and preservation of WSC content, possibly resulting from an inhibitory action on the yeasts that would consume the WSC present in sugarcane, releasing ethanol, CO_2 and water [18, 19]. According to a study [20], the ethanol produced in the silages can lead to DM losses up to 48%. Although the acetic acid concentration in these silages was greater compared with the untreated control silage, there was no effect on the population of yeast and mold. This pattern is probably due to the inhibitory effect of ethanol on yeasts population of sugarcane silage [21, 22].

In addition, those isolated strains were selected according to the highest production of acetic acid [12]. Inoculants con-

Table 4. The effects of *Lactobacillus buchneri* strains, application rate (cfu/g o fresh matter) and storage time (days) on sugarcane silage. Summary of comparisons in relation to control without inoculant application.

Study	Strain	Rate	Storage	Effects
[4]	NCIMB 40788	5×10^4	139	Lower pH, lower concentrations of ethanol, lactic acid and propionic acid, higher concentration of acetic acid, and lower DM loss
[8]	NCIMB 40788	1×10^5	92	Higher concentration of acetic acid and lower concentration of ethanol
[12]	NCIMB 40788	1×10^6	90	Lower yeast and mold population, lower lactic acid concentration and lower DM loss
[19]	UFLA 72 SIL	2.5×10^4	32	Lower yeast and higher LAB populations
[27]	NCIMB 40788	3.6×10^5	94	Lower ethanol concentration, lower yeast population and greater aerobic stability
[28]	11A44TM	1×10^5	90	Higher residual WSC, higher concentrations of acetic and propionic acids, lower ethanol concentration, and lower yeast population

taining heterofermentative LAB that produce high concentrations of acetic acid are more suitable for yeast control because of the inhibitory effect of this acid [9]. 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 [23]. 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, can lead the microorganisms to death [24]. Despite the antimicrobial activity of acetic acid, the yeast population does not change.

In sugarcane silage, the increase in ethanol concentration is normally associated with fermentation of WSC and organic acids by yeasts. However, some heterofermentative LAB, such as *L. buchneri*, can convert sugars into ethanol [25]. This could explain the increased ethanol concentration of inoculated silages with the strain 56.26.

The population of yeast and mold in the fresh sugarcane was higher than in other published studies [21]. This may be one of the reasons for the lack of inoculant effect on the population of yeast and mold. According to the study [20], silages containing a population of yeast and mold 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 substrates for yeast, mold and aerobic bacteria.

In general, the *L. buchneri* can enhance the fermentation of sugarcane silage resulting in high DM recovery and increased aerobic stability (Table **4**). 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 [11].

Inoculated silages with the strains 56.1, 56.4 and NCIMB 40788 showed better-quality fermentation than the control silage. This may also be due to a possible benefit of whole LAB population increased by inoculation [26]. Application of *L. buchneri* may have firstly increased LAB population and then LAB affected the fermentation profile resulting in high DM recovery.

As observed in other studies (Table **4**), the application of *L. buchneri* in sugarcane silage, compared to untreated silage, results in higher concentrations of acetic acid, propionic acid and WSC, lower concentrations of lactic acid and ethanol, lower yeast and mold population, lower DM loss, and greater aerobic stability. The response pattern of these works is similar to found in our study, although we did not evaluate aerobic stability.

Regarding the storage time, greater concentrations of acetic acid in silages treated with *L. buchneri* are observed from 56 d of ensiling onward [11]. In high moisture corn, [29], with inoculation of *L. buchneri,* an increase in the acetic acid concentration from the storage length of 281 d was reported. However, [30] applying *L. buchneri* on maize silage, at 14 d, any difference was not observed in concentrated acetic acid between treated and untreated control, but at 28 d, acetic acid increased and lactic acid was beginning to decrease in the *L. buchneri* treatment.

CONCLUSION

Lactobacillus 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.

CURRENT & FUTURE DEVELOPMENTS

Although there are already *L. buchneri* strains for ensiling sugarcane, the new strains evaluated in this work can improve the fermentation process speedily, thus reducing DM losses and maintaining the quality of sugarcane silage. At present, we are preparing the patent application.

LIST OF ABBREVIATIONS

CFU = Colony-forming Units

DM = Dry Matter

FM = Fresh Matter

HPLC = High-performance Liquid Chromatography

LAB = Lactic Acid Bacteria

MRS = de Man, Rogosa and Sharpe

PDA = Potato Dextrose Agar

SEM = Standard Error of Mean

WSC = Water-soluble Carbohydrates

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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