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The use of *Lactobacillus* species as starter cultures for enhancing the quality of sugar cane silage

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ABSTRACT

Sugar cane (*Saccharum* spp.) is a forage crop widely used in animal feed because of its high dry matter (DM) production (25 to 40 t/ha) and high energy concentration. The ensiling of sugar cane often incurs problems with the growth of yeasts, which leads to high losses of DM throughout the fermentative process. The selection of specific inoculants for sugar cane silage can improve the quality of the silage. The present study aimed to select strains of lactic acid bacteria (LAB) isolated from sugar cane silage and to assess their effects when used as additives on the same type of silage. The LAB strains were inoculated into sugar cane broth to evaluate their production of metabolites. The selected strains produced higher concentrations of acetic and propionic acids and resulted in better silage characteristics, such as low yeast population, lower ethanol content, and lesser DM loss. These data confirmed that facultative heterofermentative strains are not good candidates for sugar cane silage inoculation and may even worsen the quality of the silage fermentation by increasing DM losses throughout the process. *Lactobacillus hilgardii* strains UFLA SIL51 and UFLA SIL52 resulted in silage with the best characteristics in relation to DM loss, low ethanol content, higher LAB population, and low butyric acid content. Strains UFLA SIL51 and SIL52 are recommended as starter cultures for sugar cane silage.
Key words: inoculant, *Lactobacillus hilgardii*, 1,2-propanediol, yeast

INTRODUCTION

The ensiling process may occur either naturally, with epiphytic microorganisms present on the plant material, or with the addition of inoculants to improve the process, thus resulting in better quality silage. Microbial inoculants are commercially available for use in silage, and lactic acid bacteria (**LAB**) are the main microorganisms used for this purpose (Cai et al., 1999; Driehuis et al., 2001; Filya, 2003).

In general, studies with LAB inoculants show that inoculation before ensiling increases the fermentation quality of the ensiled forage (Kleinschmit and Kung, 2006; Zopollatto et al., 2009). However, the results can be inconsistent when forage crops are evaluated under different conditions, such as silo size, climate, and packing density. Factors related to the storage and application of inoculants might influence their effects on silage quality. Nevertheless, one of the determining factors for the successful application of microbial inoculants in silage is the compatibility between the plant and the microorganisms used (Muck, 2008; Ávila et al., 2009). This compatibility can be assessed by the ability of the microorganisms to use carbohydrates present in the forage and to produce metabolites of interest, primarily in the preservation of silage (e.g., acetic and lactic acids).

Sugar cane (*Saccharum* spp.) is a forage crop widely used in animal feed because of its high DM production (25 to 40 t/ha) and high energy concentration, which is due to the high concentration of sugars, mainly sucrose (250 to 300 g/kg). The ensiling of sugar cane often results in problems with the overgrowth of yeasts, which leads to high losses of DM throughout the fermentative process (Kung and Stanley, 1982). Chemical and microbiological additives have been tested with the aim of reducing yeast growth. However, microbial inoculants have produced better results than chemical additives (Carvalho et al., 2012).

Inoculants with LAB, which produce higher concentrations of acetic or propionic acids, are more suitable for yeast control because of the fungicidal effect of these acids (Moon, 1983). The addition of microorganisms that produce greater amounts of lactic acid are of interest because of their rapid effect in reducing the pH value. However, lactic acid is a potential substrate for yeast during feeding-out, reducing the aerobic stability of the silage. Inoculation with facultative heterofermentative *Lactobacillus plantarum* and obligatory heterofermentative *Lactobacillus buchneri* has been tested during ensiling of sugar cane. The results are variable, but in general, *Lb. buchneri* showed good results in reducing

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the DM losses and increased aerobic stability (Ávila et al., 2009; Roth et al., 2010). Pedroso et al. (2008) observed that *Lb. buchneri* improved fermentative and aerobic stability in silages, whereas *Lb. plantarum* strains interfered negatively in the fermentation and preservation of sugar cane silages. Ávila et al. (2010b) evaluated different LAB species (*Lb. plantarum*, *Lactobacillus paracasei*, *Lactobacillus brevis*, and *Lactobacillus brevis buchneri*) and observed that the effect of the inoculant is more related to the strain used than to the species. Inoculations with different strains that belong to the same species have resulted in silages with different characteristics, suggesting that studies should be conducted not only at the species level but also at the strain level (Saarisalo et al., 2007; Ávila et al., 2011).

The effects of microbial inoculants on the fermentation process of silage are mainly due to the production of metabolites of interest able to inhibit the growth of undesired microorganisms. Therefore, the ability of a strain to utilize different substrates present in the forage plant and to produce different metabolites can be an advantage in the competition with other microorganisms. This ability can be used as a criterion for selecting inoculants (Saarisalo et al., 2007). The present study aimed to isolate, identify, and select LAB strains for the ensiling of sugar cane by a rapid method based on the production of metabolites that are relevant for the silage process. In addition to strain performance, we evaluated the improvement of chemical and microbiological silage characteristics in experimental silos.

MATERIALS AND METHODS

Experiment 1: Isolation and Characterization of LAB from Sugar Cane Silage

Silages were made with fresh-cut sugar cane from plants that were approximately 12 mo old. The sugar cane was manually harvested and chopped using a laboratory-type chopper (PP-47, Pinheiro, Itapira, SP, Brazil) at an approximate length of 30 mm. Approximately 10 kg of chopped material was immediately conditioned in 15-L plastic buckets without valves for gas release or effluent (mini-silos). The material was compacted to a density of approximately 700 kg of fresh matter/m^3. The mini-silos were stored at room temperature (22°C) and opened after 0, 2, 15, 60, and 90 d of storage. Samples were taken on each opening day for pH analysis. Two replicates were prepared for each date of sampling.

The LAB were isolated from 80 g of sugar cane silage that was mixed with 720 mL of 0.1% sterile peptone water and homogenized in an orbital mixer for 20 min. Subsequently, 10-fold dilutions were prepared to quantify the LAB using de Man, Rogosa, and Sharpe agar (**MRS**, Difco, Detroit, MI) containing 0.1% cysteine-HCl and cycloheximide (0.4%). The plates were incubated at 30°C for 48 h under anaerobic conditions (Gas Pack Anaerobic System, BBL, Cockeysville, MD). Colonies were counted on plates with 30 to 300 well-isolated cfu, and a number of colony-forming units corresponding to the square root of the total was taken at random for identification (Holt et al., 1994). The isolates were further purified by streaking individual colonies onto MRS agar. The purified isolates were maintained at −80°C in MRS broth containing 20% (vol/vol) glycerol.

The size, shape, color, height, and edge morphology of each colony were noted. The presumptive lactobacilli were counted on MRS agar. The isolates were examined by Gram stain and for colony and cell appearance, catalase activity, motility and production of CO_2 from glucose, and gluconate in MRS broth with a Durham tube. The lactobacilli were recognized as gram-positive, catalase-negative, oxidase-negative, regular fermentative rods, and were classified as homofermentative or heterofermentative lactobacilli by their ability to produce CO_2 from glucose and gluconate.

Preselection of Bacterial Strains Based on Metabolite Production in Sugar Cane Broth. Fifty-seven isolates classified as LAB were isolated from sugar cane silage and evaluated for metabolite production. The LAB were evaluated in a 5° Brix sugar cane broth medium supplemented with 0.1% yeast extract. The Brix degree (soluble solids) was determined according to AOAC (1990) using a digital refractometer Atago PR-32 (Atago USA Inc., Bellevue, WA), with automatic temperature compensation. The broth was filtered (gauze) and sterilized (120°C, 15 min). First, the 57 strains were cultivated in MRS broth for 24 h at 35°C. After this period, the inoculum was standardized using a spectrophotometer (600 nm) at an optical density of 1.0. Subsequently, approximately 400 μL of each strain was inoculated into 300 mL of sugar cane broth, which was incubated at 35°C and 120 rpm. After 24 h of fermentation, samples of the cultures were taken to evaluate metabolite production by HPLC.

Data regarding the production of metabolites by strains were analyzed using principal component analysis (**PCA**). Sugar cane has a high concentration of soluble carbohydrates, a low buffering capacity, and DM content suitable for ensiling. Therefore, a decrease in pH occurs quickly (Kung and Stanley, 1982; Ávila et al., 2009). However, the overgrowth of yeast in sugar cane silage throughout the process causes DM losses (Kung and Stanley, 1982). Moreover, problems with deterioration after silo opening are common because of the high concentration of lactic acid, which serves as a