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Selection of tropical lactic acid bacteria for enhancing the quality of maize silage

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ABSTRACT

The objective of this study was to select lactic acid bacteria (LAB) strains isolated from silage and assess their effect on the quality of maize silage. The LAB strains were inoculated into aqueous extract obtained from maize to evaluate their production of metabolites and pH reduction. The ability to inhibit the pathogenic and silage-spoilage microorganisms' growth was evaluated. Nine LAB strains that showed the best results were assessed in polyvinyl chloride experimental silos. The inoculation of the LAB strains influenced the concentration of lactic and acetic acids and the diversity of *Listeria*. The inoculation of silages with *Lactobacillus buchneri* (UFLA SLM11 and UFLA SLM103 strains) resulted in silages with greater LAB populations and improvements after aerobic exposure. The UFLA SLM11 and SLM103 strains identified as *L. buchneri* showed to be promising in the treatment of maize silage.
Key words: inoculant, lactic acid bacteria, silage, acetic acid

INTRODUCTION

Traditionally, the most widely used forage for ensiling is the maize plant (*Zea mays* L.). In addition to its nutritional quality, maize has desirable features for ensiling, such as a high production of DM per area unit, an adequate fermentation pattern in the silo due to DM content between 28 and 40%, an adequate concentration of soluble carbohydrates, and low buffering capacity (Nussio et al., 2001). Maize silages, however, are susceptible to aerobic deterioration (Siqueira et al., 2005). Thus, the use of microbial inoculants in these silages has been recommended, with the primary goal of reducing aerobic deterioration and preserving the silage's nutritional value (Kung et al., 2003).

Spoilage and pathogenic microorganisms can be a problem in maize silages. Bacteria of the genera *Clostridium*, *Listeria*, and *Bacillus*, and the family *Enterobacteriaceae* have been reported in maize silages (Rossi and Dellaglio, 2007; Dunière et al., 2011; Konosonoka et al., 2012). Nevertheless, studies in the selection of inoculants aimed at inhibiting the growth of these deteriorating and pathogenic microorganisms are still scarce (Saarisalo et al., 2007; Marciňáková et al., 2008). Yeasts and filamentous fungi are the main microorganisms involved in the aerobic deterioration process of silages. Studies have shown that some lactic acid bacteria (**LAB**) strains can exhibit antimicrobial activity, thereby affecting many pathogenic and deteriorative microorganisms (Gollop et al., 2005).

Some authors have reported the effects of microbial inoculants on the fermentation of silages; however, few descriptions of the selection process of bacteria strains are used for this purpose. The purpose of the present study was to select LAB strains with the potential to improve the fermentative characteristics and inhibit the growth of pathogenic and spoilage microorganisms and to assess the effect of the inoculation of these strains on the nutritional value and aerobic stability of maize silages.

MATERIALS AND METHODS

Selection of Bacterial Strains for Inoculants

Lactic acid bacteria strains were originally isolated in sugar cane silage (Ávila et al., 2010a) and were preselected based on the assessment of growth and production of metabolites in the aqueous extract obtained from maize plants and their ability to inhibit the growth of pathogenic and silage-spoilage microorganisms. The aqueous extract for fermentation was obtained through chopping and grinding the entire maize plant according to Saarisalo et al. (2007). Seventy-five LAB strains were cultivated in de Man, Rogosa, Sharpe broth (M369; Himedia, Mumbai, India) for 24 h at 30°C. Subsequently, a standardization of the inoculum was performed using the number 1 standard of the McFarland scale. Then, 400 μL of each inoculum was added to 200 mL of aqueous extract from the maize plant, which was incubated at 30°C and 120 rpm for 48 h.

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To evaluate inoculum growth, decreases in pH value, and metabolite production, samples were collected immediately after inoculation and after 3, 6, 12, 24, and 48 h of fermentation. Growth was assessed by turbidimetry in a spectrophotometer at 600 nm (Shimadzu model UV-2501; Shimadzu Corp., Tokyo, Japan); a digital potentiometer was used to measure the pH, and HPLC was used to analyze metabolite production. The metabolite analysis was conducted with 24 h of fermentation in liquid phase chromatography (Shimadzu model LC-10Ai) equipped with refractive index detectors (for ethanol; model RID-10A) and UV detectors (for lactic, acetic and propionic acids; model SPD-10Ai). A cation exchange column (Shimadzu Shim-pack SCR-101H; 30 cm of length and 7.9 mm of diameter) was used, and the temperature of the column oven was 30°C for ethanol readings and 50°C for acid readings. Ultra-pure water, with its pH adjusted to 2.1 with perchloric acid, was used in the mobile phase, and the flow rate was 0.6 mL/min.

The 65 LAB strains that exhibited the best growth and efficiency in the reduction of pH were evaluated according to their ability to inhibit the growth of pathogenic and spoilage microorganisms. The pathogenic strains tested were *Clostridium perfringens* (ATCC 3624) and *Listeria monocytogenes* (ATCC 19117), and the deteriorative microorganisms were *Escherichia coli* (ATCC 11229), *Bacillus cereus* (ATCC 11778), and 9 yeast strains isolated from sugar cane silage (Ávila et al., 2010b).

Inhibition tests were performed using an agar-diffusion method according to the methodology described by Weese and Rousseau (2005), with some modifications. Pathogenic and deteriorative microorganisms were reactivated in brain heart infusion (Himedia) medium and incubated for 24 h at 37°C, except for *C. perfringens*, which was incubated under anaerobic conditions in an anaerobic chamber (Thermo Scientific, model 1025; Ottawa, Canada) for 7 d. The yeast and LAB species assessed were reactivated, respectively, on yeast extract peptone glucose (YEPG) medium [0.3% of yeast extract (Merck, Darmstadt, Germany); 0.3% malt extract (Merck); 0.5% peptone (Himedia); 1.0% glucose (Merck); 2.0% agar (Merck) per liter, containing 100 mg of chloramphenicol (Sigma-Aldrich, Milan, Italy)] and de Man, Rogosa Sharpe medium (M641I, Himedia) and incubated for 24 h at 30°C. Standardized suspensions of the growth of the deteriorative and pathogenic microorganisms assessed were prepared and compared with the McFarland 0.5 standard (approximately 1.5×10^8 cfu/mL), whereas LAB growth was standardized by comparing it with the McFarland 1 standard (approximately 3×10^8 cfu/mL).

On plates containing Mueller Hinton medium (Himedia), a superficial spread was performed using a sterile swab soaked in a suspension of the pathogenic or deteriorative microorganism to be analyzed. Subsequently, using sterile tweezers, filter-paper disks of approximately 5 mm in diameter were placed in triplicate on the medium with the suspension, to which 50 μL of the LAB suspension was added to be tested for its ability to inhibit growth of pathogenic and deteriorative microorganisms. Plates were incubated at 35°C for 24 h in aerobiosis, except for plates on which the inhibition of *C. perfringens* was assessed, which were incubated under anaerobic conditions at 35°C. Inhibition was assessed by the formation of halos around the disks, with no growth of reference pathogenic and deteriorative microorganisms. The readings of the inhibition halos were measured in millimeters using a caliper.

Molecular Identification of Selected Bacterial Strains

The strains selected for evaluation in the polyvinyl chloride (**PVC**) experimental silos were identified by DNA sequencing. Bacteria cultures were grown under appropriate conditions and collected from MRS agar plates with a sterile pipette tip and resuspended in 40 μL of PCR buffer. The suspension was heated for 10 min at 95°C and 2 μL was used as a DNA template in PCR experiments to amplify the full-length the 16S region. An approximately 1,500 bp fragment of the 16S rDNA was amplified using forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1512r (5′-ACGGCTACCTTGTTACGACT-3′; Devereux and Wilkinson, 2004). The PCR products were sequenced using an ABI3730 XL automatic DNA sequencer. The sequences were then compared with the Gen-Bank database using the BLAST algorithm (National Center for Biotechnology Information, Bethesda, MD).

Maize Ensilage in Experimental Silos

The LAB strains that exhibited the best results, including greater growth rates during fermentation, efficiency in reducing the pH, an ability to inhibit yeasts, *E. coli*, *B. cereus*, and the reference pathogens (*C. perfringens* and *L. monocytogenes*), had the best profile of VFA and lactic acid production, and the lowest ethanol production, were selected for evaluation on maize silage in PVC experimental silos. The maize, approximately 102 to 119 d old, was harvested in the rainy season using a self-propelled harvester with the particle size set to 10 mm. Inoculants were previously prepared according to Ávila et al. (2009), mixed with 80 mL of distilled water, and homogenized on 3 kg of forage to be ensiled,