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Characterization of lactic acid bacteria recovered from *atole agrio*, a traditional Mexican fermented beverage

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ABSTRACT

Our aim was to identify and characterize the lactic acid bacteria (LAB) of *atole agrio*, a fermented Mexican maize-based beverage and to evaluate whether starters could be obtained to produce it under controlled conditions. *Atole agrio* fermentation process was variable with an abundant presence of Enterobacteriaceae throughout the fermentation. Based on RAPD-PCR, *Weissella* (29.2%), *Pediococcus* (24.0%), *Lactococcus* (17.8%) and *Lactobacillus* (16.4%) were the most abundant LAB genera. Out of 88 identified LAB strains, 87.5% produced folates, 71.6% degraded phytates, 38.6% produced exopolysaccharides (EPS) and 12.5% had amylolytic activity. The majority of the strains (81.8%) were resistant to at least two of the screened nine antibiotics and 11.4% to one antibiotic. Six potential starters; *L. plantarum* IL4l1, *L. plantarum* A1MM10, *Lc. lactis* IL5l1, *Lc. lactis* A1MS3, *Leuc. pseudomesenteroides* IL5l2 and *Ped. pentosaceus* S0l10, were selected for further studies. All selected strains were phytase producers, showed antimicrobial activity and had good acidification and growth properties. In addition L. *plantarum* IL4l1, *Ped. pentosaceus* S0l10 and *Leuc. pseudomesenteroides* IL5l2 were EPS producers and had together with *Lc. lactis* IL5l1 amylolytic activity. *L. plantarum* IL4l1, *L. plantarum* A1MM10 and *Lc. lactis* IL5l1 were folate producers.

1. Introduction

Maize is economically one of the most important crops in Mesoamerica, and it has a role in the cultural and social identity of people (Sweeney, Steigerwald, Davenport, & Eakin, 2013). Maize-based foods belong to the traditional diet of indigenous populations of this region (Lorence-Quiñones, Wacher-Rodarte, & Quintero-Ramírez, 1999).

Atole agrio is a Mexican, non-alcoholic, acidic beverage derived from fermented maize. It is consumed in South-East Mexico, especially in the states of Tabasco, Chiapas and South Veracruz (Valderrama, 2012) and used by indigenous and mestizo groups for nutritional, medicinal and in ceremonial purposes. It is traditionally prepared by spontaneous fermentation in households, and raw materials, equipment and manufacturing processes differ noticeably between batches and producers leading to highly variable end products. *Atole agrio* can be prepared either by liquid or solid state fermentation (Appendix A). The end product is flavored with sugar, cinnamon or cocoa or consumed as such. Compared to other similar

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products, produced through liquid (ogi) or solid state fermentation (pozol, chorote, poto-poto), *atole agrio* manufacturing process has only few steps, maize is not boiled nor soaked prior to fermentation, the duration of fermentation is only hours instead of days, and the end product is boiled prior to the consumption (Ampe & Miambi, 2000; Castillo-Morales, Wacher-Rodarte, & Hernández-Sánchez, 2005).

Fermentation is a bioprocess that cost-efficiently improves the quality, nutritional value and organoleptic properties of perishable foods (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; De Vuyst et al., 2014). Lactic acid bacteria (LAB) are fermenting microorganisms that modify the carbohydrate content of foods, synthesize amino acids, improve the availability of B-group vitamins, degrade antinutrients, and thus increase the availability of iron, zinc and calcium (Blandino et al., 2003). In addition, LAB have antimicrobial activities against pathogens and spoilage microbes (Ouwehand & Vesterlund, 2004), and can enhance the texture, mouthfeel, taste perception and stability of fermented foods through production of exopolysaccharides (EPS) (Dal Bello, Walter, Hertel, & Hammes, 2001).

Properly selected starters can increase the nutritional value of fermented foods. For example, folate deficiency is a current problem especially in developing countries (LeBlanc et al., 2011). Folate producing LAB starters could provide a natural and economical folate source in maize-based products. Similarly, phytic acid degrading starters could enhance the bioavailability of important minerals in fermented foods (Manini et al., 2016).

The aim of our study was to identify and characterize the *atole agrio* LAB and to evaluate, whether promising starters could be obtained. Our specific interest was to screen for LAB starters that could, in the future, improve the microbiological safety of *atole agrio,* and enable the industrialization of the production of *atole agrio* and other similar products.

2. Materials and methods

2.1. Bacterial strains used as positive controls

Reference cultures used in this work were supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain), the American Type Culture Collection (ATCC, Manassas, USA), The Finnish Food Safety Authority (EVIRA, former EELA, Kuopio, Finland), the Belgian Coordinated Collection of Microorganisms (BCCM/LMG, Gent, Belgium) and Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina).

Lactobacillus rhamnosus CECT 278^T, *L. plantarum* Q8212, Q825 and Q823, *L. amylophilus* CECT 4133^T, *L. amylovorus* CECT 4132^T, *L. plantarum* ATCC 14917^T, *L. paracasei* ATCC 334 and *Lactococcus lactis* ATCC 19435^T were routinely grown on de Man, Rogosa and Sharpe Agar (MRS, LabM, Lancashire, U.K.) at 28 °C; *Listeria monocytogenes* ATCC 7644, *Salmonella* Infantis EELA 72 and *Bacillus cereus* EELA 72 on Trypticasein Soy Agar (TSA, LabM) at 37 °C; *Candida albicans* EELA 188 on Oxytetracycline Glucose Yeast Extract Agar (OGYE, LabM) at 30 °C; *Streptococcus thermophilus* LMG 18311 on M17-surcose Agar (Oxoid, Hampshire, U.K.) at 37 °C and *Bifidobacterium longum* ATCC 15707^T on MRS-cys Agar (LabM) at 37 °C.

2.2. Atole agrio manufacturing process and sampling

Three replicate *atole agrio* manufacturing processes (batch 1, 2 and 3) were performed in Mexico, state of Tabasco, city of Villahermosa. Each time *atole agrio* was prepared by both liquid and solid state and fermentation according to the traditional manufacturing processes (Appendix A). The corn cobs (60 pcs) were bought from local market (Pino Suárez) and kept at 30–40 °C overnight.

Processing of maize to *atole agrio* was done as follows: first, the grains were cut off from the corn cobs with a knife, ground and mixed with water to obtain a white dough (Valderrama, 2012). For the solid state *atole agrio* fermentation, the dough was moulded manually into balls of 100 g. Ten balls were let to ferment for 12 h at 34 °C; water (1 L) was added and maize-water slurry homogenized by hand. For the liquid fermentation, dough (750 g) was mixed with water (750 ml) prior to the fermentation and the slurry was allowed to ferment for 6 h at 34 °C. The fermentation times were selected based on the traditional manufacturing process of *atole agrio*. After either liquid or solid state fermentation the maize-water slurry was filtered and boiled (100 °C, 10 min) to achieve a thick consistency and microbiologically safer end product.

Samples for microbiological analysis and LAB recovery were taken at 0, 2, 4, 6, 12 and 24 h throughout both liquid and solid state, fermentations. In addition, samples were gathered from raw materials (grains, dough) and end products after boiling step. LAB recovery was performed until 24 h to observe, if longer fermentation changes the *atole agrio* LAB microbiota.

2.3. Microbial analysis and lactic acid bacteria isolation

Microbial counts were determined by the plate count method. Sample (25 g) was homogenized with 0.1% peptone water (225 ml) and 10-fold dilution series were prepared. Aerobic mesophilic microbes (Plate Count Agar, LabM) and Enterobacteriaceae (Violet Red Bile Glucose Agar, LabM) were incubated at 37 °C for 24 h, LAB (MRS), yeasts and molds (Potato Dextrose Agar, LabM) at 30 °C for 48 h, all colonies counted and microbial counts (log cfu g^{-1}) calculated. For each sampling time, 5–10 single colonies were randomly picked from MRS plates and sub-cultured for further analysis. Presumptive LAB or Gram-positive (Gregersen, 1978) and catalase negative bacteria (determined by transferring 359 fresh colonies from a Petri dish to a glass slide and adding H_2O_2 3%, v/v) were purified by successive sub-culturing to MRS plates. Purified isolates were stored at −70 °C in MRS broth supplemented with 20% (w/v) glycerol.

2.4. Molecular identification and clustering

Genomic DNA was extracted with Nucleo®Tissue Kit (Macherey-Nagel, Düren, Germany) using Support protocol for bacteria. The genetic diversity of 359 isolates was analyzed by Random Amplification of Polymorphic DNA (RAPD-PCR) (Plumed-Ferrer, Uusikylä, Korhonen, & von Wright, 2013) with primers P2 (5′-GAT CGG ACG G-3′), P16 (5′-TCG CCA GCC A-3′) and P17 (5′-CAG ACA AGC C-3′) (Samarzija, Sikora, Redzepovic, Antunac, & Havranek, 2002). The RAPD fingerprints recorded as digitalized images were converted, normalized, analyzed and combined using the softwares available at the corresponding laboratories: GELCOMPAR II (Applied Maths, Version 6.5, Sint-Martens-Latem, Belgium) for isolates recovered from batch 1 and 2, and the BioNumerics (Applied Maths, Version 4.61) for isolates recovered from batch 3.

RAPD dendrograms were obtained with hierarchical cluster analysis (UPGMA, Unweighted Pair Group Method with Arithmetic Mean). The similarity of band profiles was calculated based on the Pearson's correlation coefficient. At least one representative of each RAPD cluster was chosen for identification by 16S rRNA gene sequencing. For batch 1 and 2 isolates, primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 685r (5′-TCT ACG CAT TTC ACC GCT AC-3′) were used to obtain a fragment of approx. 650 bp (Plumed-Ferrer et al., 2013). PCR products were purified with NucleoSpin®Extract II kit (Macherey-Nagel) prior to sequencing (LGC Genomics GmbH, Berlin, Germany). The identification of isolates was obtained