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

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Abbreviated running headline: Lactic microbiota of *atole agrio*

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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.

Keywords: Lactic acid bacteria, Identification, Fermentation, Beverages

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 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 12h at 34°C; water (1L) was added and maize-water slurry homogenized by hand. For the liquid fermentation, dough (750g) was mixed with water (750ml) prior to the fermentation and the slurry was allowed to ferment for 6h 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 24h 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 24h 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 (25g) was homogenized with 0.1% peptone water (225ml) 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 24h, LAB (MRS), yeasts and molds (Potato Dextrose Agar, LabM) at 30°C for 48h, 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 -70ºC 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 through the GenBank DNA database using the BLAST algorithm (http://www.ncbi.nlm.mnih.gov). Sequences showing at least 99% similarity levels were accepted. The sequences of 16S rRNA gene amplicons were deposited in the GenBank database under accession numbers KX216704–KX216731. For batch 3, genomic DNA of selected isolates in each cluster was used for amplification of the almost full-length 16S rRNA gene fragment using the primers 616Valt and 630R as previously described (Elizaquível et al., 2015). The 16S rDNA sequences were used for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/) (Chun et al., 2007).

2.5 Characterization of lactic acid bacteria

2.5.1 Production of folate

Extracellular and intracellular folate production was determined according to Laiño, LeBlanc, & Savoy de Giori (2012). *L. rhamnosus* CECT 278^{T} was used as an indicator strain. Folate concentrations were expressed as mg l^{-1} of folic acid (Sigma-Aldrich, Madrid, España). All LAB characterization assays were performed in triplicate.

2.5.2 Degradation of phytates

Phytate degrading activity was screened as described by Anastasio et al. (2010) and quantified according to Kikunaga, Takahashi, & Katoh (1991).

168 **2.5.3 Exopolysaccharide production**

169 EPS production was screened in MRS Agar supplemented with 2% of glucose, lactose,
170 maltose, raffinose or sucrose (Bounaix et al., 2009). Strains with ropy colonies were
171 considered as EPS producers. *L. plantarum* Q8212, Q825 and Q823 were used as positive
172 controls.

173 **2.5.4 Amylolytic activity**

174 Amylolytic activity was screened according to Yousif et al. (2005) with minor modifications.
175 Overnight cultures of LAB strains were inoculated as three separate spots on MRS plates
176 supplemented with 1% of potato starch. After incubations (48h, 30°C; followed by 24h, 4°C)
177 iodine solution (4% v/v) was spread on top of the inoculum and the formation of clear halo
178 (mm) was observed. *L. amylophilus* CECT 4133^{T} and *L. amylovorus* CECT 4132^{T} were used
179 as positive controls.

180 **2.5.5 Antibiotic resistance**

181 Antibiotic resistance was determined according to ISO 1092 and ISO 10932:2010 standards
182 by broth dilution procedure (Korhonen, Sclivagnotis, & Wright, 2007). Minimum inhibitory
183 concentrations (MICs) of ampicillin (0.032–16 µg ml^{-1}), chloramphenicol (0.125–64),
184 clindamycin (0.032–16), erythromycin (0.016–8), gentamicin (0.5–256), kanamycin (2–
185 1024), streptomycin (0.5–256), tetracycline (0.125–64) and vancomycin (0.25–128) were
186 determined applying the cut-off values of the Committee on Antimicrobial Susceptibility
187 Testing (EUCAST, http://www.eucast.org). The control strains used were *L. plantarum*
188 ATCC 14917^{T}, *L. paracasei* ATCC 334 and *Lc. lactis* ATCC 19435^{T}. LAB were classified as
189 resistant when minimum inhibitory concentrations were higher than the recommended cut-off
190 values.

2.5.6 Antimicrobial activity

The antimicrobial activity was screened using well diffusion assay (Nikoskelainen, Salminen, Bylund, & Ouwehand, 2001). Seven Enterobacteriaceae strains, isolated and identified in a previous work from *atole agrio* (Esquivel, 2016), *Escherichia coli* S4a, S4b, S6a and S8c; *Kluyvera ascorbata* L1b, *Shigella dysenteriae* S8a and *Shig. flexneri* S8e, were used to evaluate to antimicrobial activities of *atole agrio* LAB. *L. monocytogenes* ATCC 7644, *S.* Infantis EELA 72, *B. cereus* EELA 72 and *C. albicans* EELA 188 were also included. LAB supernatants were pipetted into three replicate wells onto the TSA plates and incubated for 24h (37°C bacteria or 30°C yeast). MRS broth was used as negative and DMSO (dimethyl sulfoxide) as positive control.

2.5.7 Acidification and growth properties

Acidification and growth properties were determined according to Alfonzo et al. (2013) with minor modifications. A sterile 5 % (w/v) maize-water slurry (Risenta, Sweden) was inoculated and incubated (72h, 30°C). pH (SCHOTT, CG 842 pH meter, Germany) and microbial counts (serial dilutions on MRS) were determined at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72h.

3. Results

3.1 Characterization of spontaneous fermentation

The fermentation process of *atole agrio* was variable. This was indicated by the high standard deviations obtained from three replicate manufacturing processes (Table 1). No clear differences in the development of microbiota between liquid and solid state fermentation were observed. LAB, yeasts and molds were the dominant microbial groups. The initial counts of LAB depended highly on raw materials leading to 9.1±0.9 (liquid) and 10.2±1.3 log cfu g^{-1} (solid state fermentation), respectively. The counts of aerobic mesophilic microbes

followed the LAB counts (final counts 9.1±0.7 for liquid and 9.6±0.3 log cfu g^{-1} for solid state fermentation).

The pH value decreased from 7.0±0.7 and 6.9±0.5 to 4.7±0.5 and 4.4±0.2 in liquid and solid state fermentations, respectively, but the rate of decrease was not constant. (pH measurements were not performed for the grain, dough, and end products.) Levels of Enterobacteriaceae remained stable (7–9 log cfu g^{-1}) or decreased slightly. In the end products, discernible levels of aerobic mesophiles, LAB, yeasts, molds and Enterobacteriaceae were encountered even after the boiling step, particularly in products obtained by the liquid fermentation (Table 1).

3.2 Molecular identification and clustering

Altogether 359 LAB isolates were recovered from *atole agrio* fermentations that were taxonomically identified according to RAPD clustering and partial sequencing of the 16S rRNA gene (Illustration of RAPD-PCR patterns of batch 2 isolates is presented in Appendix B). *Weissella* (29.2%), *Pediococcus* (*pentosaceus*) (24.0%), *Lactococcus* (*lactis*) (17.8%) and *Lactobacillus* (16.4%) were the most abundant LAB genera in *atole agrio* fermentations (Table 2, Fig. 1). *Weissella* formed three distinct clusters: *W. confusa* (18.1%), *W. cibaria* (6.7%) and *W. paramesenteroides* (4.5%). *L. plantarum* (10.0%) was the most abundant *Lactobacillus* species. *Leuconostoc* (6.7%) and *Enterococcus* (5.8%) were also identified. Based on RAPD profiles and species diversity, 88 isolates were selected for further biotechnological characterization.

3.3 Characterization of lactic acid bacteria

Of the 88 LAB strains studied, the majority produced folate (Table 3). *L. plantarum* and *W. confusa* were the most efficient producers of intracellular and *L. plantarum* and *Lc. lactis* of extracellular folate. All studied LAB species included phytase active strains, but the ability of degrade phytates varied greatly within species. Activity was mainly (72.7%) intracellular

(Table 3). Sucrose induced EPS production in 38.6% of strains (Table 4). *L. plantarum, Ped. pentosaceus* and *Lc. lactis* were able to produce EPSs in the presence of other sugars. From the studied strains, 12.5% (*L. plantarum*, *Lc. lactis*, *Leuc. pseudomesenteroides* and *Ped. pentosaceus*) had amylolytic activity (Table 4).

Antibiotic resistance was observed against all studied antibiotics: 52.2% of the strains were resistant to ampicillin, 63.6% to chloramphenicol, 46.6% to clindamycin, 28.4% to erythromycin, 17.0% to gentamicin, 44.3% to kanamycin, 19.3% to streptomycin, 75.0% to tetracycline and 2.3% to vancomycin (Table 4, Appendix C). Six strains (*Lc. lactis* n=4, *Leuc. pseudomesenteroides* n=1, *W. paramesenteroides* n=1) were susceptible and ten strains (*L. brevis* n=1, *L. plantarum* n=4, *Leuc. pseudomesenteroides* n=2) and *W. confusa* n=3) resistant to all studied antibiotics.

From the six starter candidates, *L. plantarum* and *Ped. pentosaceus* were the most efficient against pathogens and spoilage microorganisms and especially against *B. cereus*, *L. monocytogenes* and Enterobacteriaceae isolated from *atole agrio* (Table 5). However, LAB strains had only marginal antimicrobial activities against *C. albicans* and *S.* Infantis (data not shown). The cell free supernatants at pH 4 showed antimicrobial activities, while none was observed with supernatants at pH 7 (data not shown). All starter candidates decreased the pH of maize-water slurry below 5.0 after 6h (Fig. 2). The best acidification and growth properties were obtained with *Lc. lactis* during the 72h fermentation.

4. Discussion

The aim of this study was to identify and characterize the LAB microbiota of *atole agrio* and to evaluate, whether promising starters could be obtained. The spontaneous fermentation of *atole agrio* proved to be variable depending on the manufacturing process (Table 1). This is most likely due to the variation in endogenous microbes present in raw materials and in the manufacturing environment. The pH value decreased in all fermentation processes at a rate

similar to other reported maize-based fermented products (Castillo-Morales et al., 2005; Elizaquível et al., 2015), but displaying great differences between replicate fermentations. The LAB counts in both liquid and solid state fermentation increased during the manufacturing process (Table 1). The concentrations of LAB, yeasts and molds were similar to or higher than those observed in other fermented maize-products (Teniola & Odunfa, 2001; Wacher et al., 2000). LAB were the dominant microbial group, but the microbial counts and metabolic activity of LAB were not sufficient to prevent the growth of Enterobacteriaceae (Table 1). The abundance of Enterobacteriaceae during *atole agrio* fermentation is most probably caused by the high ambient temperature (30–40°C) and humidity. Acid-resistant *E. coli* pathogenic strains have also been isolated from another maize-fermented food, pozol (Sainz et al., 2001). Even though Enterobacteriaceae should be destroyed during the boiling step, their metabolites can cause unpleasant sensory properties, such as bitter, pungent and manure-like flavours (Westling et al., 2016). Due to the unpleasant organoleptic properties and food safety risks linked to Enterobacteriaceae, their presence at the end product is not desired.

While the most prevalent LAB genus in liquid fermentation was *Pediococcus* (29.8%), *Weissella* (36.3%) dominated in solid state fermentation (Table 2, Fig. 1). LAB species diversity of the solid state fermentation was slightly greater compared to liquid fermentation. *Ent. asini*, *Ent. casseliflavus*, *L. coryniformis* and *L. dextrinicus* were only present in solid state fermentation. At the end of liquid fermentation (6h) *Pediococcus*, *Lactococcus* and *Lactobacillus* were equally present whereas at the end of solid state fermentation (12h), *Lactococcus* was the major LAB genus. Previously, *L. plantarum*, *L. fermentum*, *L. delbrueckii*, *Leuc. lactis*, *Leuc. mesenteroides*, *Ent. faecium*, *Ped. pentosaceus* and *W. confusa* have been reported as the dominant LAB genera in other Latin American and African fermented maize products (Ampe & Miambi, 2000; Elizaquível et al., 2015;

290 Hounhouigan, Nout, Nago, Houben, & Roumbouts, 1993). In our study the isolation of LAB
291 was done from MRS plates (preferentially selecting rod-shaped LAB). It is unknown,
292 whether, for example, M17 medium would have recovered more coccoid LAB species.
293 The production of folate and phytase activity were screened to obtain starters that could
294 enhance the nutritional value of fermented products. *Ent. mundtii*, *Lc. lactis*, *L. plantarum*, *L.*
295 *pentosus*, *Leuc. pseudomesenteroides*, *Ped. acidilactici* and *Str. thermophilus* have been
296 reported as active folate producers (LeBlanc, Savoy de Giori, Smid, Hugenholtz, & Sesma,
297 2007; Salvucci, LeBlanc, & Pérez, 2016). In our study, also *L. brevis*, *Ped. pentosaceus*, *W.*
298 *confusa* and *W. paramesenteroides* produced both extra- and intracellular folate (Table 3).
299 Phytase activity was widespread among all *atole agrio* LAB, as reported previously (De
300 Angelis et al., 2003; Manini et al., 2016). *L. plantarum* and *Ped. pentosaceus* have been
301 reported as the major EPS producers similarly to our study (Manini et al., 2016). Amylolytic
302 activity has been observed in *S. infantarius* from pozol and in *Lactobacillus* strains isolated
303 from fermented products (Díaz-Ruiz, Guyot, Ruiz-Teran, Morlon-Guyot, & Wacher, 2003;
304 Reddy, Altaf, Naveena, Venkateshwar, & Kumar, 2008). Also in our study, some LAB
305 strains possessed amylolytic activity. While mature maize is used to make pozol and other
306 fermented foods, tender maize ("de dobla"), which contains more simple carbohydrates, is
307 used to prepare *atole agrio*. This may be the reason for the limited occurrence of amylolytic
308 LAB within this product.
309 The antibiotic resistance of *atole agrio* LAB was high and specially in *Weissella* (Table 4,
310 Appendix C). Because of the antibiotic resistance of the *Weissella* strains tested, and due to
311 the fact that *Weissella* do not have Qualified Presumption of Safety (QPS) status (Abriouel et
312 al., 2015; EFSA, 2017), all *Weissella* strains were rejected as starters, although they are
313 present in spontaneously fermented *atole agrio* (Table 2, Fig. 1). Moreover, the widespread

and high antibiotic resistances amongst *Weissella* indicate a need to re-evaluate the MIC cut-off values for this LAB genus (Table 4, Appendix C).

Considering the short duration of *atole agrio* fermentation and high microbial counts obtained from spoilage microbes, starter possessing antimicrobial activities and sufficient growth and acidification properties would have a potential to improve the microbial safety of atole agrio. Six LAB strains were screened for their antimicrobial activities, acidification and growth properties based on promising results in biotechnological properties and antibiotic resistance assays (Table 3, Table 4). In our study, the antimicrobial activities were apparently based on the acidic environment induced by LAB (Table 5). *L. plantarum* and *Ped. pediococcus* have shown to be specially antilisterial, similarly to our results (Manini et al., 2016). Interestingly all the seven *atole agrio* derived Enterobacteriaceae strains tested were sensitive to *atole agrio* LAB, but Enterobacteriaceae survived until the end of *atole agrio* fermentations (Table 1). This observation implies that the levels of LAB are not high enough in the beginning of the spontaneous fermentation to inhibit the growth of Enterobacteriaceae. In general, only antibiotic susceptible strains are accepted as starters. However, due to the lack of antibiotic susceptible strains and the fact that the end product is thoroughly boiled at the end of manufacturing process, we have chosen both antibiotic susceptible LAB strains and strains possessing only marginal antibiotic resistances. Ultimately, six LAB strains, *L. plantarum* IL4l1, *L. plantarum* A1MM10, *Lc. lactis* IL5l1, *Lc. lactis* A1MS3, *Leuc. pseudomesenteroides* IL5l2 and *Ped. pentosaceus* S0l10 (Table 3, Table 4, Table 5, Fig. 2, Appendix C) were chosen as starters. All strains were phytase producers, showed antimicrobial activity and had good acidification and growth properties. *L. plantarum* IL4l1, *Ped. pentosaceus* S0l10 and *Leuc. pseudomesenteroides* IL5l2 were also EPS producers and had together with *Lc. lactis* IL5l1 amylolytic activity. *L. plantarum* IL4l1, *L. plantarum* A1MM10 and *Lc. lactis* IL5l1 were folate producers.

5. Conclusions

The identification and characterization of LAB microbiota present in *atole agrio* is reported in this work. This research gives valuable information regarding LAB indigenous microbiota. Since the microbial densities varied greatly within *atole agrio* fermentations, and Enterobacteriaceae were present in the end products regardless of the boiling procedure, there is a need for a well-defined starter to control the fermentation process. In addition, a well-adapted starter enabled a successful fermentation by reducing the period of fermentation and by speeding up the acidification. The traditional manufacturing process of *atole agrio* is short (6–12h) and ambient temperature is high. It is extremely important to decrease the numbers of undesirable competing microorganisms, commonly present in maize spontaneous fermentation, to ensure the food safety of *atole agrio*.

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Conflict of interest:

The authors report no conflict of interest.

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Table 1 Microbial counts (log cfu g^{-1})[a] and pH[a] (average±SD) during the liquid and solid state *atole agrio* manufacturing processes.

Table 2 Identification of recovered isolates and distribution of species along liquid and solid state *atole agrio* manufacturing processes.

Table 3 Production of folates [a,b] and degradation of phytates [a,b] of *atole agrio* LAB strains. Strains chosen for further studies are highlighted in grey.

Table 4 Exopolysaccharide production properties[a,b], amylolytic activity properties[a,b] and antibiotic properties[a,b] resistances of *atole agrio* LAB strains. Strains chosen for further studies are highlighted in grey.

Table 5 Antimicrobial activities[a,b] of the six starter candidates against *B. cereus, E. coli, Kl. ascorbata, L. monocytogenes, Shigella dysenteriae* and *Shigella flexneri*. pH of the LAB cell free supernatants was 4.

Figure 1 Distribution of LAB genera among liquid and solid state *atole agrio* manufacturing processes: ▨ *Enterococcus,* ▭ *Lactobacillus* ■ *Lactococcus,* ▩ *Pediococcus,* ▩ *Leuconostoc* and ▬ *Weissella*.

Figure 2 Acidification (pH) and growth properties (log cfu g^{-1}) of the six most promising *atole agrio* LAB strains (average±SD) in 5 % (w/v) maize flour water slurry at 30°C. Values are means of triplicates. ━ *L. plantarum* IL4l1, ━ ━ *L. plantarum* A1MM10, ▪ ▪ ▪ *Lc. lactis* IL5l1, ▬ *Lc. lactis* A1MS3, ▬ ▬ *Leuc. pseudomesenteroides* IL5l2, ▪ ▪ ▪ *Ped. pentosaceus* S0l10 and ▬ spontaneous fermentation. The log cfu g^{-1} of spontaneous fermentation remained as 0 during 72h.

Appendix A The traditional manufacturing process of *atole agrio* (Valderrama 2012).

Appendix B Dendrograms corresponding to RAPD-PCR patterns of *atole agrio* LAB isolates from batch 2 of the three replicate fermentations with three primers. Upper line shows the percentage of similarity.

520 **Appendix C** Antibiotic resistance[a,b] of *atole agrio* LAB strains. Resistance to antibiotics

521 highlighted with grey.

	Liquid fermentation							Solid state fermentation							
Time point [h]	grains*	dough*	0h	2h	4h	6h	end product	0h	2h	4h	6h	8h	10h	12h	end product
Aerobic mesophiles	6.3±1.2	6.4±0.0	8.6±2.2	12.1±0.0	8.2±0.7	9.1±0.7	3.2±1.6	7.9±0.9	7.8±0.5	8.6±0.8	9.1±0.1	10.6±3.5	8.9±0.6	9.6±0.3	0
Lactic acid bacteria	6.4±0.6	7.0±1.1	7.5±1.9	8.2±0.4	8.6±0.4	9.1±0.9	4.0±0.6	8.1±0.3	7.4±0.5	7.7±0.4	10.1±0.0	9.4±0.8	10.0±0.5	10.2±1.3	0
Yeasts and molds	6.2±0.7	7.3±0.9	7.3±0.7	7.8±1.2	9.0±1.6	8.6±1.1	4.2±0.0	7.5±0.9	8.3±1.0	8.2±0.5	8.9±0.4	8.9±1.1	7.9±1.2	9.1±0.6	0
Enterobacteriaceae	6.3±0.4	6.8±0.3	7.2±0.2	8.0±0.5	7.9±0.4	7.8±0.4	1.7±1.0	7.7±0.8	7.7±0.1	8.2±0.6	8.5±0.7	8.4±1.2	8.5±1.0	8.3±1.5	3.2±0.0
pH	na	na	7.0±0.7	6.7±0.4	5.7±1.1	4.7±0.5	na	6.9±0.5	6.8±0.7	6.3±0.8	5.7±0.7	4.9±0.2	4.4±0.4	4.4±0.2	na

* raw materials

na not analyzed

a Results are means of triplicates.

		Liquid fermentation						Solid state fermentation						
Time [h]	RM*	0	2	4	6	12	24	0	2	4	6	12	24	Total (%)
N° Isolates	52	42	31	34	36	7	11	27	28	26	34	15	16	359 (100)
Enterococcus	**6**		**1**	**3**	**4**	**1**		**3**	**2**			**1**		**21 (5.8)**
Ent. asini									2					2 (0.6)
Ent. casseliflavus	1							2						3 (0.8)
Ent. faecium	4		1	3	4	1		1				1		15 (4.2)
Ent. mundtti	1													1 (0.3)
Lactobacillus	**14**	**8**	**2**	**3**	**10**	**2**	**2**	**7**	**3**	**1**	**2**	**2**	**3**	**59 (16.4)**
L. brevis	3				10			1						14 (3.9)
L. coryniformis													1	1 (0.3)
L. dextrinicus									1					1 (0.3)
L. mali	3					2			1			1		7 (1.9)
L. plantarum	8	8	2	3			2	6	1	1	2	1	2	36 (10.0)
Lactococcus	**5**	**5**	**8**	**7**	**9**	**2**			**1**	**5**	**14**	**8**		**64 (17.8)**
Lc. lactis	5	5	8	7	9	2			1	5	14	8		64 (17.8.)
Leuconostoc	**5**	**3**	**1**	**3**	**1**				**5**		**5**	**1**		**24 (6.7)**
Leuc. pseudomesenteroides	5	3	1	3	1				5		5	1		24 (6.7)
Pediococcus	**8**	**11**	**10**	**4**	**12**	**2**	**9**	**8**	**7**	**2**	**1**		**12**	**86 (24.0)**
Ped. pentosaceus	8	11	10	4	12	2	9	8	7	2	1		12	86 (24.0)
Weissella	**14**	**15**	**9**		**14**			**9**	**10**	**18**	**12**	**3**	**1**	**105 (29.2)**
W. cibaria	1	3	3	3				2		2	7	3		24 (6.7)
W. confusa	9	11	6	11				6	2	14	5		1	65 (18.1)
W. paramesenteroides	4	1						1	8	2				16 (4.5)

* raw materials (maize grains and dough)

LAB strain	Production of folate		Degradation of phytates		
	EC [ng ml^{-1}]	IC [ng ml^{-1}]	Halo [mm]	EC [ng ml^{-1}]	IC [ng ml^{-1}]
L. brevis					
A5LM7	3.3	20.2	7	-	131.8
A5LMS1	1.0	19.2	7	-	103.7
A5LY5	3.2	19.6	7	-	123.9
A2SY8	1.1	25.2	6	-	79.3
L. mali					
BTl5	6.0	15.5	8	-	243.0
L. dextrinicus					
S2l3	-	-	-	-	266.3
L. plantarum					
A1M5	10.3	31.8	8	-	131.8
A1M10	8.0	33.8	-	-	-
A1MM4	0.2	7.6	-	-	-
A1MM10	11.9	40.0	7	-	131.4
A1Y6	0.5	14.1	6	-	79.0
A1Y8	10.9	34.3	6	-	81.3
A2LY9	0.3	6.1	7	-	154.0
A2SMS4	10.4	40.5	8	-	122.4
A2SMM4	9.8	28.5	7	-	101.5
A2SY9	0.9	4.1	7	-	109.4
A3LM8	0.2	13.2	5	-	76.9
A3SY3	9.4	29.1	5	-	79.8
A4SMM9	11.2	39.1	4	-	66.6
A5SMS5	-	-	8	-	130.6
A9LM2	8.5	32.3	6	-	93.3
A9LMM9	7.8	24.9	8	-	131.5
A9SMS5	0.6	2.8	6	-	84.3
IIS10l2	20.8	19.9	11	-	273.4
IL4l1	16.9	15.5	14	266.3	12.2
Lc. lactis					
A1MS3	10.3	37.3	9	-	130.8
A2MLS5	16.2	31.9	-	-	-
A3SMS5	-	-	-	-	-
A5SMS6	11.1	38.7	-	-	-
GTl1	11.1	19.9	15	-	278.3
IIS4l2	19.0	20.9	16	-	254.1
IL5l1	15.2	18.5	16	-	284.2
IS6A1	13.3	16.3	16	-	258.2
L3l7	6.8	20.6	16	-	264.9
Leuc. pseudomesenteroides					
A1MM5	8.8	34.7	7	-	131.8
A2LMS2	7.9	23.2	7	-	122.8
A3LMM4	0.7	0.7	7	-	120.4
A3SY8	0.3	14.3	5	-	72.9
A4LY1	7.1	24.4	-	-	-
A4LY4	13.0	29.3	-	-	-
A5SY8	-	-	-	-	-
IL5l2	-	11.3	11	-	270.2
S6l4	5.3	23.8	10	-	265.4
Ped. pentosaceus					
A1MS4	8.1	31.3	7	-	73.4
A1Y1	1.7	23.5	-	-	-
A2SMS1	3.2	23.1	-	-	-
A2SMS6	3.2	23.1	-	-	-
A3LMM8	9.9	36.8	-	-	-
A3LMM9	5.5	22.9	-	-	-
A3SY6	10.4	20.1	-	-	-
A4LM10	3.6	20.3	7	-	126.9

A4LY2	-	-	7	-	108.2
A4SMS2	-	-	8	-	73.9
A4SMS4	7.4	33.5	-	-	-
A9LMM1	0.0	6.0	8	-	114.8
A9LMS9	3.7	26.8	-	-	-
A9SM2	10.1	36.8	-	-	-
A9SM7	0.4	21.9	6	-	100.1
S0l8	-	-	11	-	261.4
S0l10	-	-	11	-	253.4
S4l8	-	-	11	-	252.1
W. cibaria					
IIL4l3	3.3	2.1	12	-	275.9
IL3l3	6.3	20.5	12	-	266.9
L4l2	1.40	4.40	12	-	246.9
W. confusa					
A1M2	11.2	41.3	6	-	108.2
A1M9	1.5	25.1	5	-	76.7
A2LM9	11.8	36.7	6	-	114.8
A2LMM10	11.6	38.4	7	-	130.6
A2SM1	10.9	38.5	-	-	-
A3LM6	11.9	35.1	6	-	121.3
A3SM8	10.4	37.9	4	-	62.8
A4LM6	10.5	26.9	6	-	100.1
A4LMM9	0.4	23.7	7	-	103.7
A5SM5	0.2	10.6	4	-	73.4
A4SM7	9.9	40.8	7	-	131.4
A4SM8	8.6	32.0	6	-	126.9
A4SM10	11.0	40.0	-	-	-
A4SY2	10.9	36.3	-	-	-
A5SM10	9.1	28.7	7	-	123.8
A9SMM9	12.1	35.9	-	-	-
W. paramesenteroides					
A1M10	10.6	37.8	7	-	130.8
A1SMS10	8.5	38.2	7	-	154.0
A2LMM8	4.3	22.5	-	-	-
A3SMS3	10.7	37.2	4	-	73.9
A3SMS10	3.9	34.7	-	-	-
A4SMM6	11.8	37.1	-	-	-
S0l7	-	-	11	-	262.5
S2l6	-	-	11	-	260.4

[EC] extracellular

[IC] intracellular

[-] activity not detected

[a] Results are means of triplicates.

[b] Standard deviations were always lower than 10% of the means.

LAB strain	Exopolysaccharide production					Amylolytic activity	Antibiotic resistance
	Glucose	Lactose	Maltose	Raffinose	Sucrose		
L. amylophilus							
CECT 4133^T	na	na	na	na	na	+	na
L. amylovorus							
CECT 4132^T	na	na	na	na	na	+	na
L. brevis							
A5LM7	-	-	-	-	-	-	R
A5LMS1	-	-	-	-	-	-	R
A5LY5	-	-	-	-	-	-	R
A2SY8	-	-	-	-	-	-	R
Lact. mali							
BTl5	-	-	-	-	+	-	R
L. dextrinicus							
S2l3	-	-	-	-	-	-	R
L. plantarum							
A1M5	-	-	-	-	-	-	R
A1M10	-	-	-	-	-	-	R
A1MM4	-	-	-	-	-	-	R
A1MM10	-	-	-	-	-	-	R*
A1Y6	-	-	-	-	-	-	R
A1Y8	-	-	-	-	-	-	R
A2LY9	-	-	-	-	-	-	R
A2SMS4	-	-	-	-	.	-	R*
A2SMM4	-	-	-	-	-	-	R
A2SY9	-	-	-	-	-	-	R
A3LM8	-	-	-	-	-	-	R
A3SY3	-	-	-	-	-	-	R
A4SMM9	-	-	-	-	-	-	R
A5SMS5	-	-	-	-	-	-	R
A9LM2	-	-	-	-	-	-	R
A9LMM9	-	-	-	-	-	-	R
A9SMS5	-	-	-	-	-	-	R
IIS10l2	+	+	+	+	+	+	R
IL4l1	-	-	+	+	+	+	R*
Q8212						na	na
Q823	+	+	+	+	+	na	na
Q825	+	+	+	+	+	na	na
Lc. lactis							
A1MS3	-	-	-	-	-	-	S
A2MLS5	-	-	-	-	-	-	S
A3SMS5	-	-	-	-	-	-	S
A5SMS6	-	-	-	-	-	-	S
GTl1	-	-	-	-	-	+	R*
IIS4l2	-	+	-	-	+	+	R*
IL5l1	-	-	-	-	-	+	R*
IS6A1	-	-	-	-	-	+	R*
L3l7	-	-	-	-	-	+	R*
Leuc. pseudomesenteroides							
A1MM5	-	-	-	-	-	-	R
A2LMS2	-	-	-	-	-	-	R
A3LMM4	-	-	-	-	-	-	R
A3SY8	-	-	-	-	+	-	R
A4LY1	-	-	-	-	-	-	R
A4LY4	-	-	-	-	+	-	R
A5SY8	-	-	-	-	+	-	R
IL5l2	-	-	-	-	+	+	S
S6l4	-	-	-	-	+	-	R*
Ped. pentosaceus							
A1MS4	-	-	-	-	-	-	R

A1Y1	-	-	-	-	-	-	R
A2SMS1	-	-	-	-	-	-	R*
A2SMS6	-	-	-	-	-	-	R*
A3LMM8	-	-	-	-	-	-	R
A3LMM9	-	-	-	-	-	-	R
A3SY6	-	-	-	-	+	-	R
A4LM10	-	-	-	-	-	-	R
A4LY2	-	-	-	-	-	-	R*
A4SMS2	-	-	-	-	-	-	R
A4SMS4	-	-	-	-	-	-	R
A9LMM1	-	-	-	-	-	-	R
A9LMS9	-	-	-	-	-	-	R
A9SM2	-	-	-	-	-	-	R*
A9SM7	-	-	-	-	-	-	R
S0l8	-	-	+	+	+	+	R
S0l10	+	-	+	-	+	+	R
S4l8	-	-	-	+	+	+	R*
W. cibaria							
IIL4l3	-	-	-	-	+	-	R
IL3l3	-	-	-	-	+	-	R
L4l2	-	-	-	-	+	-	R
W. confusa							
A1M2	-	-	-	-	+	-	R
A1M9	-	-	-	-	+	-	R
A2LM9	-	-	-	-	-	-	R
A2LMM10	-	-	-	-	+	-	R
A2SM1	-	-	-	-	+	-	R
A3LM6	-	-	-	-	+	-	R
A3SM8	-	-	-	-	-	-	R
A4LM6	-	-	-	-	+	-	R
A4LMM9	-	-	-	-	+	-	R
A5SM5	-	-	-	-	+	-	R
A4SM7	-	-	-	-	+	-	R
A4SM8	-	-	-	-	+	-	R
A4SM10	-	-	-	-	+	-	R
A4SY2	-	-	-	-	+	-	R
A5SM10	-	-	-	-	+	-	R
A9SMM9	-	-	-	-	+	-	R
W. paramesenteroides							
A1M10	-	-	-	-	-	-	R
A1SMS10	-	-	-	-	-	-	R
A2LMM8	-	-	-	-	-	-	R
A3SMS3	-	-	-	-	-	-	R
A3SMS10	-	-	-	-	-	-	R
A4SMM6	-	-	-	-	+	-	R
S0l7	-	-	-	-	+	-	S
S2l6	-	-	-	-	-	-	R

[R] resistance to two or more of the tested antibiotics

[R*] resistance to one of the tested antibiotics, S susceptible to all antibiotics tested

[-] activity not detected

[na] not analyzed

[a] Results are means of triplicates.

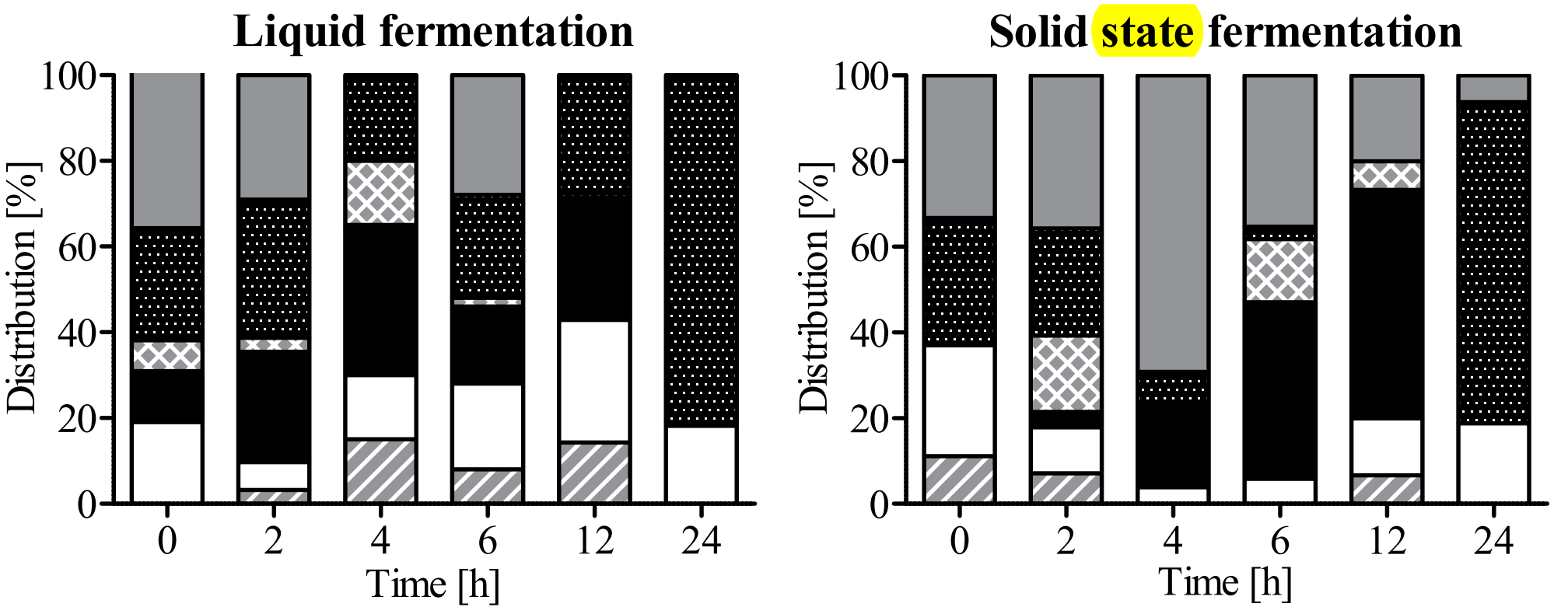
[b] Standard deviations were always lower than 10% of the means.

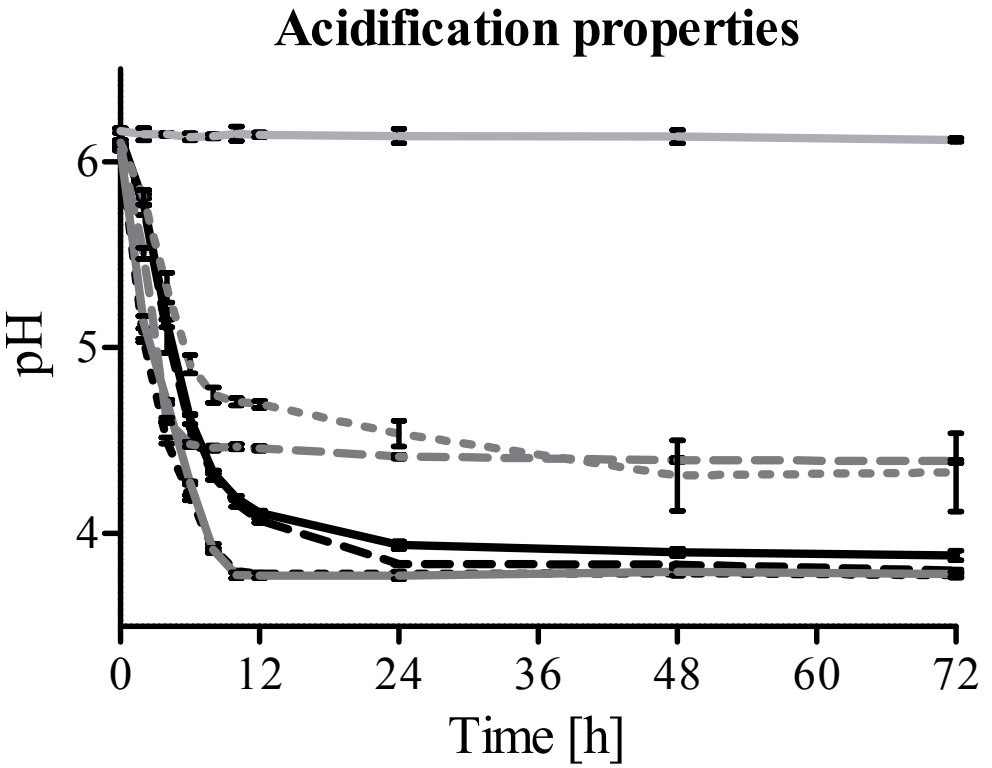
LAB strain	*B. cereus* EELA 72	*E. coli* S4a	*E. coli* S4b	*E. coli* S6a	*E. coli* S8c	*Kl. ascorbata* L1b	*L. monocytogenes* ATCC 7644	*Shig. dysenteriae* S8a	*Shig. flexneri* S8e
L. plantarum IL4l1	++	+	+	+	+	+	++	+	++
L. plantarum A1MM10	+	+	+	+	+	+	+	+	+
Lc. lactis IL5l1	-	-	+	-	-	-	+	-	+
Lc. lactis A1MS3	-	-	+	+	-	+	+	+	+
Leuc. pseudomesenteroides IL5l2	-	+	-	-	-	+	-	-	-
Ped. pentosaceus S0l10	+	+	+	+	+	-	+	+	+

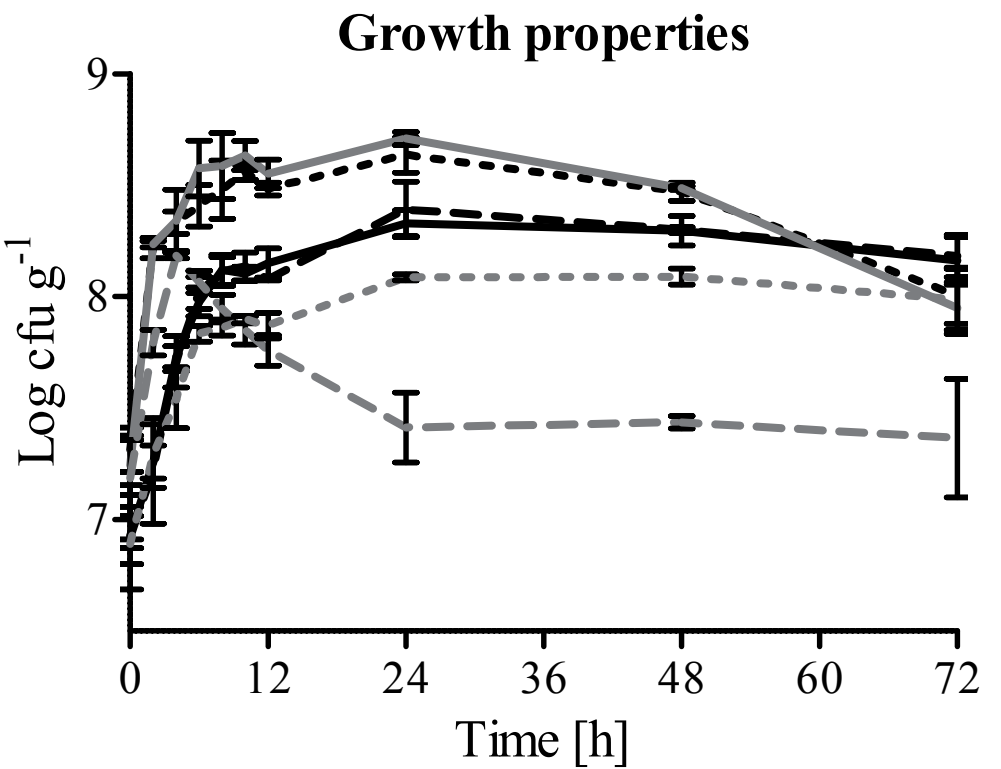
$^{-}$ Length of inhibition zone 0–2 mm; $^{+}$ length of inhibition zone >2–4 mm; $^{++}$ length of inhibition zone >4 mm.

[a] Results are means of triplicates.

[b] Standard deviations were always lower than 10% of the mean.


Liquid fermentation
Solid state fermentation
Distribution [%]
100
80
60
40
20
0
0
2
4
6
12
24
Time [h]


Acidification properties
pH
6
5
4
0
12
24
36
48
60
72
Time [h]


Growth properties
Log cfu g-1
9
8
7
0
12
24
36
48
60
72
Time [h]

Highlights:

1. The lactic acid microbiota of *atole agrio* was characterized for the first time.
2. The microbiota of *atole agrio* was variable between batches.
3. *Atole agrio* LAB had antimicrobial activity against Enterobacteriaceae.
4. Folate producers and phytate degrading LAB were identified.
5. Promising LAB starters were selected.