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Technological properties of Lactic acid bacteria isolated from raw cereal material

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ABSTRACT

Lactic Acid Bacteria (LAB) were isolated from cereals and seeds from Argentinean markets. Colonies representing different morphological appearances were isolated and differentiated based on phenotypic characteristics. Fifty strains were identified by 16S rRNA gene sequencing analysis obtaining LAB from genera *Enterococcus*, *Lactobacillus* and *Pediococcus*. Technological and nutritional characteristics (acidifying capacity, antimicrobial production, proteolytic activity, folate production) were analyzed. *Lactobacillus pentosus* ES124 and *Lactobacillus plantarum* ES137 presented high production of folate (61 and 57 ng/mL, respectively) as did *Enterococcus mundtii* ES63 which reached a total folate production of 70 ng/mL. Six LAB strains produced bacteriocin-like inhibitory substances (BLIS) with antilisterial activity. Eight strains were selected for use as sourdough starters and *in situ* applications based on their important technological properties.

1. Introduction

Lactic acid bacteria (LAB) have been used in the production of fermented foods and beverages for centuries. They contribute to the flavor, microbial safety, enhancement of shelf-life, improvement of texture and sensory profile of the final products (Axelsson & Ahrné, 2000; Leroy & De Vuyst, 2004). LAB are considered safe for health and they have received the GRAS (Generally Recognized As Safe) status by the US Food and Drug Administration. Production of metabolites by LAB such as acetic acid, ethanol, aromatic compounds, bacteriocins, exopolysaccharides, and several enzymes are technologically interesting (Axelsson & Ahrné, 2000; Leroy & De Vuyst, 2004; Mayo et al., 2010; Patel & Prajapati, 2013) and explain why LAB are used in food to produce a wide variety of fermented products (Leroy & De Vuyst, 2004; Paucean, Vodnar, Socaci, & Socaciu, 2013). Food safety can be improved using LAB that can decrease pH through the production of many organic acids such as lactic, acetic and propionic as end products. Futhermore, some LAB can produce bacteriocins or bacteriocin like substances (BLIS) which are ribosomally synthesized, extracellular released short peptides or proteins with bactericidal or bacteriostatic activity on closely related species (Collins, Cotter, Hill, & Ross, 2010; Cotter, Hill, & Ross, 2005; Nes, Diep, & Holo, 2007; Salvucci, Saavedra, & Sesma, 2007). They have attracted great interest in terms of food safety because of their capacity to prevent the growth of food-borne pathogens (Chen & Hoover, 2003; de Carvalho et al., 2010; Eijsink et al., 2002; Gautam & Sharma, 2009; Jordan et al., 2014; Salvucci, Hebert, Sesma, & Saavedra, 2010).

Growth in high salt media is another desirable property for starter cultures since NaCl is one of the most important additives for preserving food. LAB are capable of growing in elevated NaCl concentrations which allow them to participate in fermentative processes in the absence of harmful or undesirable bacteria that cannot grow in the presence of salts (Chikthimmah, Anantheswaran, Roberts, Mills, & Knabel, 2001).

Proteolytic activity allows LAB to degrade peptides and proteins and generate different metabolites that contribute to flavor, antimicrobial activity, and structure of different foods (Gänzle, Loponen, & Gobbetti, 2008). The use of vitamin-producing microorganisms, especially LAB is a natural and economically viable alternative to fortification with chemically synthesized pseudo-

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vitamins (LeBlanc et al., 2013; Leblanc, Pía Taranto, Molina, & Sesma, 2010). Searching for indigenous LAB strains capable of producing folate would allow the production of foods with elevated concentrations of folates that are less likely to cause undesirable side effects such as masking vitamin B12 deficiencies or the deactivation of certain liver enzymes (Laiño, Juarez del Valle, Savoy de Giori, & LeBlanc, 2014; Laiño, Leblanc, & Savoy de Giori, 2012).

Studying LAB can be difficult, especially in raw materials that are subjected to uncontrolled conditions. Although cereals and flours contain the nutrients required for the growth of nutritionally fastidious LAB, its low water content and the dormant state in which LABs are found, makes their isolation very difficult (Alfonzo et al., 2013). Well-designed strategies such as enrichment are required to improve LAB isolation from these ecological niches.

In this work we report the isolation, characterization and identification of LAB from cereals and seeds. Also, the evaluation of some technological properties and the selection of some candidates to be included as starters for the production sourdoughs or novel foods are provided.

2. Materials and methods

2.1. Isolation procedure and 16S rDNA identification

Wheat (commercial cultivars Biointa 3004, Klein Tauro, Klein Tigre, Klein Guerrero, Atlax, Esmeralda), Sorghum (cultivars Malón, Paisano and Argensor), and triticale, all sown on large parts of Argentina, and Oat, Rye, Chia, Sesame were obtained from markets in Argentina and used as raw materials. Enrichment was performed by placing the materials (5% w/v) in de Man Rogosa Sharpe (MRS) broth containing 5% maltose or in LAPTg (peptone 1.5%; tryptone 1%; glucose 1%; yeast extract 1%; Tween 80 1%) broth for 48 h at 30 °C. Also commercial wholemeal flour were used as raw material. Isolation of LAB was performed by spontaneous fermentation mixing 20 g of flour with 20 ml of sterile water. This slurry was propagated for ten days according to Corsetti et al. (2007) to obtain LAB enrichment.

All the samples, after enrichment were serially diluted in peptone water (peptone 0.1%, NaCl 0.9%) and plated on the corresponding media and incubated microaerophilically for 48 h.

Genotypic characterization of selected LAB was carried with 16S rDNA gene sequencing. PCR and DNA sequencing was performed by MACROGEN Inc. (Korea). The resultant sequences were compared to sequenced bacteria with a BLAST search using the GenBank/EMBL/DDBJ database.

2.2. Bacteria and growth conditions

All potential LAB were grown in MRS media containing 5% (w/v) maltose or LAPTg broth when the microscopic morphology of their colonies were either bacilli or cocci, respectively. *Pediococcus* and *Enterococcus* strains were grown in LAPTg at 37 °C. Indicator strains were grown as follows: *Listeria (L.) innocua* ATCC33090 was grown in Brain Heart Infusion (BHI, Britania, Argentina) at 37 °C. *Lactobacillus (Lb.) helveticus* ATCC15807, *Lb. reuteri* ATCC23272, *Lb. reuteri* BP83 and *Lb. plantarum* ATCC8014 were grown in MRS at 37 °C. *Escherichia coli* and *Staphylococcus (S.) aureus* were grown in BHI at 37 °C. Growth in the corresponding media in the presence of 6.5% (w/v) NaCl was examined. All strains were maintained at −20 °C in their appropriate media with 15% glycerol.

2.3. Acidification activity

To evaluate the kinetics of acidification by LAB in vitro, sterile flour extract (SFE) broth was prepared following previously described methods (Alfonzo et al., 2013). Briefly, 200 g of wheat flour (humidity: 13.8%; protein: 9.7%) was suspended in 1 L distilled water and sterilized by autoclaving at 121 °C for 20 min; the flour was then precipitated and removed; and the supernatant was used as liquid broth in subsequent experiments. Overnight LAB cultures, grown in MRS or LAPTg (according to the isolation procedure), were harvested by centrifugation at 5000 g for 5 min, washed with NaCl 0.9% and, to standardize bacterial inoculum, suspended in fresh solution to an optical density at 600 nm of 1.00, corresponding to approximately 10^9 CFU per mL, as measured with a Biotraza 721 Spectrophotometer (China). The acidifying capacity of LAB was subsequently assayed during their incubation in 20 ml of SFE at 30 °C with 1% (v/v) of the solution consisting of the cell suspension. pH measurements were made every 2 h for the first 8 h of incubation and after 24 and 48 h.

2.4. Proteolytic activity

Proteolysis was assayed against gelatin on agar plates, using a modified method (Vermelho et al., 1996). Briefly, a loopful of the culture to be evaluated was inoculated on agar plates with the detection medium (2% (w/v) sucrose (Merck), 0.5% (w/v) yeast extract (Britania), 2% (w/v) peptone (Britania), 1.5% (w/v) agar (Britania) autoclaved and supplemented with 1% (w/v) gelatin) and incubated at 37 °C for 48 h. Extracellular protease detection was done after staining agar plates with Coomassie blue (0.25%, w/v) for 1 h in methanolacetic acid-water 5:1:4 (v/v/v) and destaining with methanolacetic acid-water. Regions of enzyme activity were detected as clear areas, indicating that hydrolysis of the substrates had occurred.

2.5. Folate determination

After growth in MRS or LAPTg broth, strains were washed 3 times with saline solution (0.85% wt/v NaCl), resuspended in this solution at the original culture volume, and used to inoculate at 4% (v/v) folic acid casei medium (FACM, Difco, USA) that were then incubated without agitation at 30 °C for 18 h. After growth, this washing–resuspension procedure was repeated, and the resulting LAB suspension was used to inoculate at 2% (v/v) the respective fresh vitamin-free media. This last step was repeated 7 times with the cultures showing good growth (observed by increased turbidity); strains that did not grow in vitamin-free media were not used in further studies. After the last incubation, 2 samples were taken to determine the concentration of extracellular and intracellular folates. A sample (500 μL) of LAB-containing vitamin-free media was taken, and 500 μL of folate protecting buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1.5% (w/v) ascorbic acid) to prevent vitamin oxidation and degradation was added, mixed and centrifugated for 5 min at 5000 g. The supernatant was collected (extracellular sample) and the pellet was resuspended in 500 μL of protecting buffer (intracellular sample). Both samples were then boiled (100 °C) for 5 min, centrifuged for 6 min at 10 000 g, and stored at −70 °C until used for vitamin determinations.

Folate concentrations were determined by a previously described microbiological assay using *Lb. rhamnosus* NCIMB 10463 as the indicator organism (Laiño et al., 2012). Briefly, diluted samples or different concentrations of HPLC-grade folic acid (Fluka BioChemica, Sigma–Aldrich, Switzerland) were placed with the indicator strain and incubated statically during 48 h at 37 °C in 96-well sterile microplates containing the folate-free medium (Difco, USA). The optical density was read at 580 nm (OD580) using a microplate reader (VERSAmax tuneable microplate reader, Molecular Devices, USA). The folate concentration of the samples was determined by comparing the OD with those obtained with the standard curve prepared using commercial folic acid.