LWT - Food Science and Technology 70 (2016) 185–191

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Technological properties of Lactic acid bacteria isolated from raw cereal material

Emiliano Salvucci [a, b, *], Jean Guy LeBlanc [c], Gabriela Pérez [a, d]

[a] ICYTAC-CONICET-UNC, Juan Filloy s/n, Universidad Nacional de Córdoba, 5000, Córdoba, Córdoba, Argentina
[b] Laboratorio de Microbiología y Biotecnología, Departamento de Química Industrial y Aplicada, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, 5000, Córdoba, Argentina
[c] CERELA-CONICET, Chacabuco 145, 4000, Tucumán, Argentina
[d] Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, 5000, Córdoba, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form
5 February 2016
Accepted 19 February 2016
Available online 23 February 2016

Keywords:
Lactic acid bacteria
Folate
Bacteriocin
Cereals
Wheat flour

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-

* Corresponding author. ICYTAC-CONICET-UNC, Juan Filloy s/n, Universidad Nacional de Córdoba, 5000, Córdoba, Córdoba, Argentina.
E-mail address: salvucci.emiliano@gmail.com (E. Salvucci).

http://dx.doi.org/10.1016/j.lwt.2016.02.043
0023-6438/

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.

2.6. Antimicrobial activity

Assays for antibacterial activity were performed by the double layer diffusion test. Cell-free supernatants (CFS) from overnight LAB cultures grown in MRS or LAPTg were harvested by centrifugation at 5000 g for 5 min. Supernatants were neutralized (pH = 6) with 0.1% NaOH in order to avoid inhibitory action of pH. An agar plate was over-layed with 5 ml semi-solid BHI agar containing 10 μl of a culture of the indicator microorganism (10^8 CFU/ml). *L. innocua* ATCC33090, *S. aureus, Escherichia coli, Lb. plantarum* ATCC8014, *Lb. reuteri* BP83, *Lb. brevis* ATCC14869, *Lb. reuteri* ATCC23272, *Lb. helveticus* ATCC15807 were used as indicator strains. Also, *Pediococcus (P.) acidilactici* ES51, *Enterococcus mundtii* ES198, *Lb. plantarum* ES147, *Pediococcuspentosaceus* ES110 and *Lb. pentosus* ES124 isolated in this work, were used as indicator strains. Each supernatant (10 μl) was spotted onto the agar plates and incubated overnight at 37 °C. The formation of an inhibition halo around the spotted material indicated a positive antagonistic result. To determine the heat resistance of antimicrobial compounds, inhibition assay after heat treatment (120 °C, 20 min) was performed.

The proteinaceous nature of BLIS was determined by well diffusion assay method. 30 μL of Trypsin (1 mg/mL) and Proteinase K (1 mg/mL) were poured in a well next to a well containing active CFS. The diffusion of protease produced the inactivation of BLIS and the resulting truncation of the inhibition halo occurred after incubation overnight at 37 °C.

The amount of bacteriocin produced was assayed from overnight cultures. Arbitrary Units per milliliter (AU/mL) were determined as follow: two-fold serial dilutions of neutralized supernatants were spotted (10 μL) on agar lawn inoculated with *L. innocua* ATCC33090. AU were calculated considering the last dilution that produce inhibition (n) as a clear halo and referring to in milliliters: 2^n (1000/x), where x is the volume of bacteriocin used in the assay.

2.7. Statistical analysis

All values were expressed as means ± standard deviations (SD). Statistical analyses were performed with the software package SigmaPlot for Windows version 12.0 (Systat Software Inc., Chicago Illinois, USA) using ANOVA GLM, followed by a Tukey's posthoc test, and differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. LAB isolation

All the enrichment assays showed pH values near 4.5 after 48 h incubation. Colonies (n = 350) were isolated from the different raw material described in section 2.1. Most of the isolates were shown to be cocci with different appearance (diplococci, chains, and tetramers). From these microscopic observations and macroscopic evaluations of colonies, 50 strains (41 cocci and 9 bacilli) were selected based on the fact that they had different morphologies and possessed gram positive and catalase-negative phenotypes. These 50 strains were the identified by genotypic analysis (see Table 1) and different technological properties were studied in order to select the most promising starter cultures.

3.2. Acidification power

From the 50 identified bacteria, 10 strains (*E. mundtii* ES62, ES70 and ES251, *Enterococcus faecium* ES78, ES169 and ES213, *Lb. fermentum* ES140 ES142 and ES148, and *Enterococcus gallinarum* ES244) were able to lower the pH of the sterile flour extract (SFE) broth below 4 after 8 h of incubation. After 24 h, *Lb. pentosus* ES124, *Lb. fermentum* ES137 and *Lb. fermentum* ES148 showed the lowest pH values reaching 3.30, 3.23 and 3.27, respectively (Fig. 1). This rapid acidification potential is important in the elaboration of fermented foods were lower pH values are desired not only for organoleptic properties but also in order to prevent the growth of pathogenic microorganisms.

3.3. Proteolytic activity and growth at 6.5% NaCl

Seven (7) strains of lactobacilli and enterococci were able to degrade gelatin in plate assays as observed by the formation of a clear halo after incubation on a medium containing gelatin, which indicates proteolytic activity of these strains (Table 1). Also, most of the strains evaluated (near 88%) were able to grow in the presence of 6.5% NaCl (Table 1); the main exceptions were those from the species *Lb. fermentum, Lb. brevis* and *P. pentosaceus*. The resistance of the strains of interest is also very important since these could be used in the preparation of different foods that contain elevated concentrations of salt.

3.4. Folate production

Only 40 strains were screened for folate production since 10 were not able to grow in folate-free culture medium after 7 subcultures. The highest total amount of folate were obtained with *E. mundtii* ES63 (71 ng/mL) and ES32 (62 ng/mL), *Lb. pentosus* ES124 (62 ng/mL) and *Lb. plantarum* ES137 (57.3 ng/mL). *P. acidilactici* ES22 produced 56 ng/mL and it is another candidate to be used in food products (Table 1). *E. mundtii* ES63 and *Lb. pentosus* ES124 were the highest intracellular folate producers reaching 34 ng/mL and 33 ng/mL whereas strains *E. muntii* ES31 and ES42 shown the highest levels of extracellular folate (39 ng/mL each). From these results, many strains were shown to be very good folate producers. Folate production was different among the strains, not only quantitatively but also in its cellular location. This opens the door to the elaboration of different folate enriched foods where the vitamin could either be released in the food product or be protected inside the cells.

3.5. Antimicrobial activity

Eight (8) selected strains showed some type of antimicrobial activity against the indicators strains (Table 2). *Lb. pentosus* ES124 and *E. mundtii* ES151 showed a slight inhibition against *S. aureus* in well assays. This inhibition seems to be because of the low pH since the neutralization of the supernatants resulted in the elimination of the inhibitory activity. *E. mundtii* ES151 and ES198, and *E. faecium* ES194, ES195, and ES216 showed antimicrobial activity against *Listeria* and *Pediococcus*. The peptidic nature of these compounds were characterized in a plate assay by co-diffusion after trypsin and proteinase K treatments (Fig. 2). Antilisterial activity persists after neutralization. Furthermore, this antimicrobial activity does not disappear after heat treatment (120 °C, 20 min) demonstrating that the selected strains are in fact bacteriocin producers.

E. faecium ES216 also inhibited *Lb. plantarum* and *E. mundtii* ES198, another bacteriocin producer. This wider spectra of action suggests that ES216 could produce a different type of bacteriocin that the others or that it could produce more than one bacteriocin. *P. pentosaceus* ES51 has shown to be a highly sensitive indicator strain to bacteriocins.

The amount of bacteriocin produced was 1×10^5 and 2×10^5 UA/mL depending on the producer strain *L. innocua* was used as indicator microorganism (Table 3). The identification of bacteriocin producing strains is important to elaborate safer foods where

Table 1
Folate production, proteolytic activity and growth in presence of 6.5% NaCl of LAB strains.

Strain	Source	Folate (ng/ml)						6.5% NaCl	Proteolytic activity*
		Extracellular	SD	Intracellular	SD	Total	SD		
Enterococcus mundtii ES4	Wheat Klein Tigre	2.3	0.0	29.1	0.3	31.3	0.4	+	+
Lactobacillus pentosus ES11	Wheat Klein Tauro	7.9	0.0	30.1	0.4	37.9	0.4	+	−
Lactobacillus pentosus ES13	Wheat Klein Tauro	13.3	0.2	30.1	0.2	43.4	0.4	+	−
Enterococcus mundtii ES14	Wheat Klein Tauro	14.9	0.1	28.7	0.4	43.6	0.5	+	−
Pediococcus acidilactici ES22	Oat	27.1	1.0	28.7	0.3	55.8	1.3	+	−
Enterococcus faecalis ES28	Oat	9.7	0.1	37.1	2.6	46.9	2.6	+	−
Enterococcus mundtii ES31	Wheat Klein Tauro	12.8	0.2	39.3	3.1	52.1	3.4	+	−
Enterococcus mundtii ES32	Wheat Klein Tauro	28.4	2.4	33.7	1.4	62.1	3.8	+	−
Pediococcus acidilactici ES34	Wheat Klein Tauro	5.3	0.1	33.3	1.4	38.6	1.5	+	−
Pediococcus acidilactici ES42	Wheat Klein Guerrero	14.1	0.0	39.0	1.5	53.0	1.5	+	−
Pediococcus acidilactici ES43	Wheat Klein Guerrero	13.4	0.9	28.2	0.3	41.6	1.2	+	−
Enterococcus mundtii ES45	Wheat Klein Guerrero	ND	ND	ND	ND	ND	ND	+	−
Pediococcus acidilactici ES51	Wheat Klein Tigre	13.4	0.7	27.0	0.2	40.3	0.9	+	−
Enteroccus mundtii ES53	Wheat Klein Tigre	14.8	1.2	29.2	0.7	44.1	1.9	+	−
Pediococcus acidilactici ES54	Wheat Klein Tigre	14.9	0.1	37.6	1.1	52.5	1.2	+	−
Enterococcus mundtii ES62	Wheat Klein Tigre	15.5	0.3	35.3	2.2	50.8	2.4	+	−
Enterococcus mundtii ES63	Wheat Klein Tigre	34.8	1.4	36.1	1.5	70.9	2.9	+	−
Enterococcus mundtii ES70	Wheat Klein Tauro	4.1	0.0	18.3	0.4	22.4	0.4	+	−
Enterococcus faecium ES71	Sesame	12.5	0.4	36.8	0.6	49.4	1.0	+	−
Enterococcus faecium ES74	Sesame	15.2	0.4	36.2	0.9	51.4	1.3	+	−
Enterococcus gallinarum ES75	Sesame	22.5	0.6	32.3	0.4	54.8	1.1	+	−
Enterococcus faecium ES78	Chia	18.5	0.9	27.1	1.3	45.6	2.2	+	+
Enterococcus durans ES87	Chia	14.7	0.3	9.0	0.3	23.7	0.6	+	−
Pediococcus pentosaceus ES110	Wholemeal flour	18.7	0.6	33.0	1.4	51.7	2.0	−	−
Lactobacillus fermentum ES111	Wholemeal flour	6.9	0.5	28.2	2.7	35.2	3.2	−	−
Lactobacillus pentosus ES124	Wholemeal flour	33.3	1.1	28.5	0.3	61.8	1.4	+	−
Lactobacillus fermentum ES129	Wholemeal flour	14.5	1.0	36.6	0.7	51.1	1.8	+	−
Lactobacillus plantarum ES137	Wholemeal flour	24.1	0.9	33.2	0.1	57.3	0.9	−	−
Lactobacillus fermentum ES140	Wholemeal flour	20.0	0.6	29.3	0.1	49.3	0.7	+	−
Lactobacillus fermentum ES142	Wholemeal flour	6.8	0.2	29.2	0.3	36.0	0.6	+	−
Lactobacillus plantarum ES147	Wholemeal flour	28.9	0.1	1.8	0.1	30.7	0.1	−	+
Lactobacillus fermentum ES148	Wholemeal flour	3.6	0.0	2.1	0.0	5.8	0.1	−	+
Enterococcus mundtii ES151	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus mundtii ES153	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus faecium ES169	Wheat Biointa 3004	8.3	0.1	21.3	1.9	29.6	2.1	+	−
Enterococcus mundtii ES173	Rye	ND	ND	ND	ND	ND	ND	+	−
Enterococcus mundtii ES174	Rye	17.1	0.7	32.9	0.0	50.0	0.7	+	−
Enterococcus gallinarum ES188	Wheat Atlax	16.8	0.5	19.9	0.3	36.6	0.8	+	−
Enterococcus sp. ES193	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus faecium. ES194	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus faecium. ES195	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus mundtii ES198	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus faecium ES213	Oat	14.8	0.5	22.9	0.3	37.8	0.8	+	−
Enterococcus faecium ES216	Oat	ND	ND	ND	ND	ND	ND	+	+
Enterococcus faecium ES224	Wheat Esmeralda	ND	ND	ND	ND	ND	ND	+	−
Enterococcus mundtii ES243	Wheat Esmeralda	4.6	0.1	32.6	2.2	37.1	2.3	+	−
Enterococcus gallinarum ES244	Sesame	18.9	0.6	30.5	1.0	49.4	1.6	+	−
Enterococcus mundtii ES250	Oat	6.8	0.8	31.2	3.6	38.0	4.3	+	−
Enterococcus faecium ES251	Rye	8.1	0.0	32.2	0.4	40.3	0.4	+	−
Lactobacillus brevis ES253	Rye	7.3	0.0	33.9	1.7	41.3	1.7	−	−

ND: not determinate; +, Positive for protease production or positive growth; -, negative for protease production or non-growth.

pathogens growth could be inhibited.

Based on the combination of all the above mentioned results of the technological and functional properties (acidification power, salt tolerance, proteolityc activity, antimicrobials and folate production), the eight (8) best LAB strains were selected for further development of a fermented food as listed in Table 4.

4. Discussion

Raw cereal materials constitute an interesting ecological niche to find new strains of LAB. These bacteria can improve the technological and nutritional properties of fermented foods. Diversity of LAB is interesting not only at a species level, but also at strain level, because most properties are strain dependent. Based on the Gram positive, catalase negative and morphology, 50 strains were selected and submitted to technological assays. Eleven strains were able to reduce the pH of SFE below 4 after 8 h at 30 °C. This property is interesting to select the potential strain to be used in sourdough and to obtain breads with better rheological and flavor properties.

In this work 8 strains of *Enterococcus* that produce bacteriocins were identified. These substances have shown an antimicrobial power (2×10^5 AU/mL) similar or higher than very known strong antimicrobial compounds like enterocin CRL35 (5.9×10^4 UA/mL) (Salvucci et al., 2007), pediocin PA-1 (3.3×10^4 UA/mL) (Piva & Headon, 1994), munticina KS (1.2×10^4 UA/mL) (Kawamoto et al., 2002) and nisin (1.27×10^4 AU/mL). The activity spectrum of these BLIS suggests that they could be a type II bacteriocin, according to the current classification (Heng, Wescombe, Burton, Jack, & Tagg, 2007). Its main property is an antagonist activity against *Listeria*. *L. monocytogenes* is a foodborne pathogen that causes a severe disease in humans and represents a major concern in food industries (Hernandez-Milian & Payeras-Cifre, 2014).

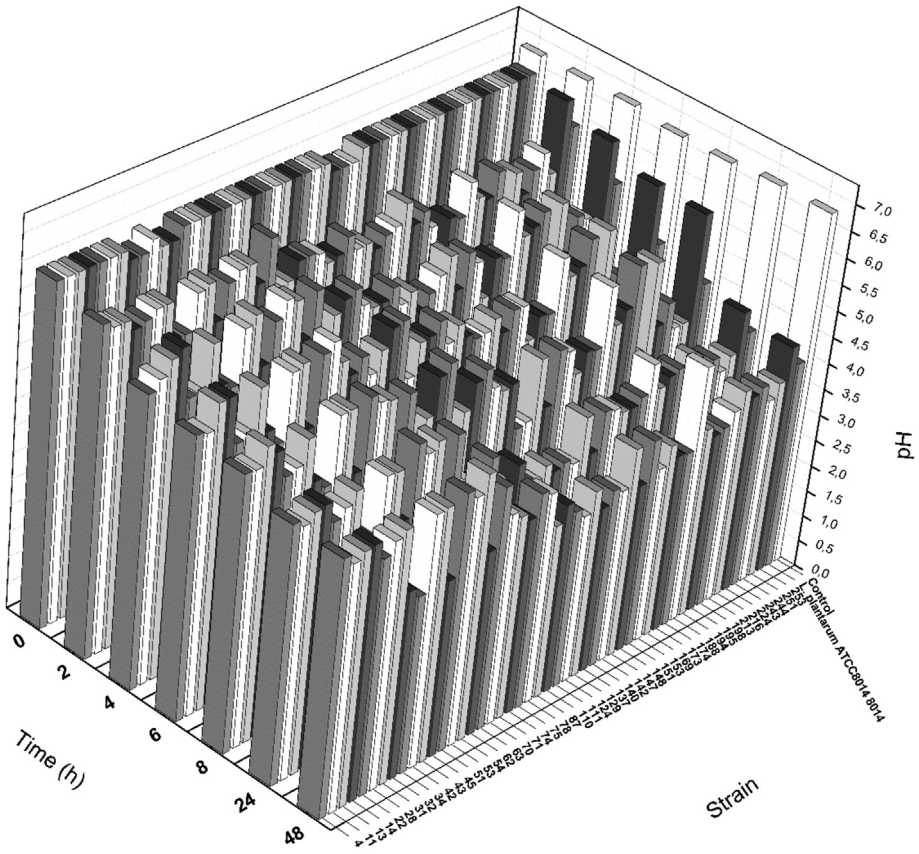

Fig. 1. Kinetics of acidification of wheat flour by LAB.

Table 2
Antimicrobial activity of different strains against indicator strains.

		Indicator strain												
		P. acidilactici ES51	*E. mundtii* ES198	*Lb. plantarum* ES147	*P. pentosaceus* ES110	*Lb. pentosus* ES124	*Lb. helveticus* ATCC15807	*Lb. reuteri* ATCC23272	*Lb. reuteri* BP83	*Lb. plantarum* ATCC8014	*L. innocua* ATCC 33090	*E. coli*	*S. aureus*	*Lb. brevis* ATCC14869
Producer strain	ES124	+	−	−	−	−	−	−	−	−	−	−	+	−
	ES147	−	−	−	−	−	−	−	−	−	+	−	−	−
	ES151	+	−	−	+	−	−	−	−	−	+	−	+	−
	ES153	−	−	−	−	−	−	−	−	−	+	−	−	−
	ES194	−	−	−	−	−	−	−	−	−	+	−	−	−
	ES195	+	−	−	+	−	−	−	−	−	+	−	−	−
	ES198	+	−	−	+	−	−	−	−	−	+	−	−	−
	ES216	+	+	+	+	−	−	−	−	−	+	−	−	−

+:inhibition; −:no inhibition.

Further studies are necessary to characterize and identify these peptides. Bacteriocins are cationic, amphipathic peptides usually with low molecular weight (rarely over 10 kDa), which have post-translational modifications and can be easily degraded by proteolytic enzymes. They have no secondary effects because of their degradation in the gastrointestinal tract (Yang, Lin, Sung, & Fang, 2014). Based on their antimicrobial potential, these strains could be used to improve the safety of fermented products. Furthermore, bacteriocins are of great interest due to a trend toward active and green packaging. The use of biomaterials including cellulose, starch, pectin and poly(lactic acid) (PLA) mixed with these antimicrobial for food packaging is an advanced field of research and application in the food industry (Blanco Massani, Botana, Eisenberg, & Vignolo, 2013). The combination of biodegradability of films with the antimicrobial property of bacteriocin will be of full benefit in an active packaging in order to extend shelf-life of different products. Our lab is currently working on this issue.

Folate producing strains are very interesting to improve the nutritional benefits of different foods. However, folate production by endogenous bacteria in cereals remains scarcely studied. Only a few strains have been studied for this capacity in oat brans and rye sourdoughs (Herranen et al., 2010; Kariluoto et al., 2006). In this study, 40 strains isolated from a wide range of cereals and seeds (such as different cultivars of wheat, sorghum, oat, rye, chia and sesame) where shown to increase folate concentrations in a strain dependent manner. The production of folate was highest in strain *E. mundtii* ES32 and ES63, *Lb. pentosus* ES124 and *Lb. plantarum* ES137. *Enterococcus* are normally considered poor folate producers; however *E. mundtii* ES32 and ES63 can produce 62 and 70 ng/mL of total folate which is similar to the levels obtained by folate

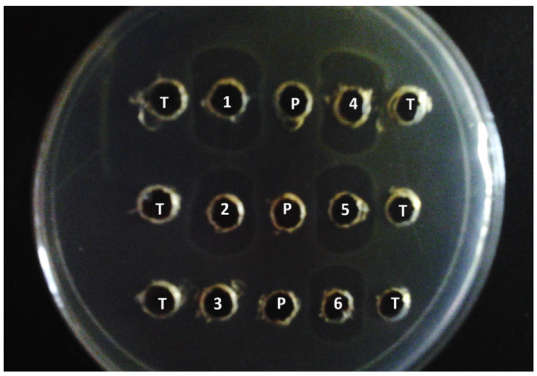

Fig. 2. Inhibition halos and protein nature of BLIS. Numbered wells contain different neutralized CFS (1:ES195; 2:ES153; 3:ES197; 4:ES216; 5:ES198; 6:ES194). Next wells contains Trypsin (T) or Proteinase K (P). Inhibition halos are truncated or deformed because of the co-diffusion of proteases and bacteriocin from the next wells. The degradation of bacteriocin result in no inhibition of *Listeria*. ES197 is a not bacteriocin producer strain.

Table 3
Amount of antimicrobial produced by producer strains. Listeria innocua ATCC33090 was used as indicator strain.

Strain	Dilution (n)	AU/mL $\left[2^n\left(\frac{1000}{10}\right)\right]$
ES151	10	102400
ES153	10	102400
ES194	10	102400
ES195	11	204800
ES198	11	204800
ES216	11	204800

producing lactobacilli (LeBlanc et al., 2011). Moreover, *Lb. plantarum* ES137 produced higher concentrations of folate than those recently reported by strains of this species (Laiño et al., 2014). *Lb. plantarum* is known to be folate producer and it is the only lactobacilli that has the complete enzymes necessary for the shikimate pathway for chorismate production necessary for folate production (Masuda et al., 2012; Rossi, Amaretti, & Raimondi, 2011). *Lb. plantarum* is also common bacteria in sourdough. *P. acidilactici* ES22 reached 56 ng/mL of folate production. Because of their ability to produce high levels of folate these strains mentioned could be used to produce sourdoughs to obtain bread with increased folate levels (Rossi et al., 2011). Also, they can be used to develop starters for fermented milks with high vitamin content (LeBlanc et al., 2013).

From all of 50 isolates from wheat, wholemeal and seed raw materials, 8 LAB strains based on technological properties. These strains were selected for further studies in order to develop applications of these strains to improve the nutritional properties and safety of food products.

Table 4
Selected strain based on their technological properties.

Selected strain	Technological property
E. mundtii ES32	Folate producer (62 ng/mL) – Salt tolerance
E. mundtii ES63	Folate producer (71 ng/mL) – Salt tolerance
P. acidilactici ES22	Folate producer (52 ng/mL) – Salt tolerance – Proteolytic activity
Lb. pentosus ES124	Folate producer (62 ng/mL) – Acidifier (pH 3,3 in 8 h) – Salt tolerance
Lb. fermentum ES148	Proteolytic activity – Acidifier (pH 3,3 in 8 h) – Salt tolerance
Lb. plantarum ES137	Folate producer (57 ng/mL) –Acidifier
E. mundtii ES198	Bacteriocin production (2048 UA/mL) – Salt tolerance
E. faecium ES216	Bacteriocin production (2048 UA/mL), activity against LAB and *Listeria* – Salt tolerance – Proteolytic activity.

5. Conclusion

Cereal raw material is an interesting source of LAB. We selected eight strains of LAB from different raw material based on vitamin production, acidifying power, proteolysis and antimicrobial activity. *E. mundtii* ES32 and ES63, *Lb. pentosus* ES124 and *Lb. plantarum* ES137 are folate producers. *Lb. pentosus* ES124 is a powerful acidifier strain and a good vitamin producer that allow us to postulate and evaluate it as a sourdough starter. It could be combined with another selected bacteriocin producer strain since it is resistant to all bacteriocins evaluated in inhibition assays. *E. faecium* ES216 produces the most powerful bacteriocin with activity against Listeria and furthermore has shown proteolytic activity. It could decrease the pH of SFE to 4 in 24 h. This is other selected strain to be evaluated in sourdough production. Another interesting strain is *Lb. fermentum* ES148 which has high acidification power and proteolytic activity. *P. acidilactici* ES22 has shown proteolytic activity and salt tolerance. *E. mundtii* ES198 is a bacteriocin producer strain. These factors allow us to postulate these strains of different species of LAB as potential starters in sourdough production and to be used their antimicrobial peptides to improve the safety of the final products.

Acknowledgments

The authors would like to thank Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP-CONICET 2012-2014 N°11220110101051, Ministerio de Ciencia y Tecnología (MINCYT) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) (PICT 2012- 1050) for the financial support. We would also like to thank to Instituto Superior de Investigación, Desarrollo y Servicios en Alimentos (ISIDSA) and Phytopathology lab from Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, where part of this work was performed. Finally, the author would like to thank the Universidad Nacional de Córdoba.

References

Alfonzo, A., Ventimiglia, G., Corona, O., Di Gerlando, R., Gaglio, R., Francesca, N. … Settanni, L. (2013). Diversity and technological potential of lactic acid bacteria of wheat flours. *Food Microbiology, 36*(2), 343–354. http://doi.org/10.1016/j.fm.2013.07.003.

Axelsson, L., & Ahrné, S. (2000). Lactic acid bacteria. In F. G. Priest, & M. Goodfellow (Eds.), *Applied microbial systematics* (pp. 367–388). Springer Netherlands. Retrieved from http://link.springer.com/chapter/10.1007/978-94-011-4020-1_13.

Blanco Massani, M., Botana, A., Eisenberg, P., & Vignolo, G. (2013). Development of an active wheat gluten film with Lactobacillus curvatus CRL705 bacteriocins and a study of its antimicrobial performance during ageing. *Food Additives & Contaminants: Part A, 31*(1), 164–171. http://doi.org/10.1080/19440049.2013.859398.

de Carvalho, K. G., Bambirra, F. H., Kruger, M. F., Barbosa, M. S., Oliveira, J. S., Santos, A. M. … Franco, B. D. (2010). Antimicrobial compounds produced by Lactobacillus sakei subsp. sakei 2a, a bacteriocinogenic strain isolated from a Brazilian meat product. *Journal of Industrial Microbiology and Biotechnology, 37*(4), 381–390.

Chen, H., & Hoover, D. (2003). Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety, 2*(3), 82–100. http://doi.org/10.1111/j.1541-4337.2003.tb00016.x.

Chikthimmah, N., Anantheswaran, R. C., Roberts, R. F., Mills, E. W., & Knabel, S. J. (2001). Influence of sodium chloride on growth of lactic acid bacteria and subsequent destruction of Escherichia coli O157:H7 during processing of Lebanon bologna. *Journal of Food Protection, 64*(8), 1145–1150.

Collins, B., Cotter, P. D., Hill, C., & Ross, P. (2010). Applications of lactic acid bacteria-produced bacteriocins. In F. Mozzi, R. R. Raya, & G. M. Vignolo (Eds.), *Biotechnology of lactic acid bacteria* (pp. 89–109). Wiley-Blackwell. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/9780813820866.ch5/summary.

Corsetti, A., Settanni, L., Chaves López, C., Felis, G. E., Mastrangelo, M., & Suzzi, G. (2007). A taxonomic survey of lactic acid bacteria isolated from wheat (Triticum

durum) kernels and non-conventional flours. *Systematic and Applied Microbiology, 30*(7), 561–571. http://doi.org/10.1016/j.syapm.2007.07.001.
Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology, 3*(10), 777–788. http://doi.org/10.1038/nrmicro1273.
Eijsink, V. G. H., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., & Nes, I. F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek, 81*(1–4), 639–654. http://doi.org/10.1023/A:1020582211262.
Gänzle, M. G., Loponen, J., & Gobbetti, M. (2008). Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. *Trends in Food Science & Technology, 19*(10), 513–521. http://doi.org/10.1016/j.tifs.2008.04.002.
Gautam, N., & Sharma, N. (2009). Bacteriocin: safest approach to preserve food products. *Indian Journal of Microbiology, 49*(3), 204–211. http://doi.org/10.1007/s12088-009-0048-3.
Heng, N. C. K., Wescombe, P. A., Burton, J. P., Jack, R. W., & Tagg, J. R. (2007). The diversity of bacteriocins in gram-positive bacteria. In P. D. M. A. Riley, & D. M. A. Chavan (Eds.), *Bacteriocins* (pp. 45–92). Springer Berlin Heidelberg. Retrieved from http://link.springer.com/chapter/10.1007/978-3-540-36604-1_4.
Hernandez-Milian, A., & Payeras-Cifre, A. (2014). What is new in listeriosis? *BioMed Research International, 2014*, 358051 http://doi.org/10.1155/2014/358051.
Herranen, M., Kariluoto, S., Edelmann, M., Piironen, V., Ahvenniemi, K., Iivonen, V. … Korhola, M. (2010). Isolation and characterization of folate-producing bacteria from oat bran and rye flakes. *International Journal of Food Microbiology, 142*(3), 277–285. http://doi.org/10.1016/j.ijfoodmicro.2010.07.002.
Jordan, K., Dalmasso, M., Zentek, J., Mader, A., Bruggeman, G., Wallace, J. … Malakauskas, M. (2014). Microbes versus microbes: control of pathogens in the food chain. *Journal of the Science of Food and Agriculture, 94*(15), 3079–3089. http://doi.org/10.1002/jsfa.6735.
Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L., & Piironen, V. (2006). Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. *International Journal of Food Microbiology, 106*(2), 137–143. http://doi.org/10.1016/j.ijfoodmicro.2005.06.013.
Kawamoto, S., Shima, J., Sato, R., Eguchi, T., Ohmomo, S., Shibato, J., … Sameshima, T., et al. (2002). Biochemical and genetic characterization of mundticin KS, an antilisterial peptide produced by Enterococcus mundtii NFRI 7393. *Applied and Environmental Microbiology, 68*(8), 3830–3840.
Laiño, J. E., Juarez del Valle, M., Savoy de Giori, G., & LeBlanc, J. G. J. (2014). Applicability of a Lactobacillus amylovorus strain as co-culture for natural folate bioenrichment of fermented milk. *International Journal of Food Microbiology, 191*, 10–16. http://doi.org/10.1016/j.ijfoodmicro.2014.08.031.
Laiño, J. E., Leblanc, J. G., & Savoy de Giori, G. (2012). Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. *Canadian Journal of Microbiology, 58*(5), 581–588. http://doi.org/10.1139/w2012-026.
LeBlanc, Laiño, J. e., del Valle, M. J., Vannini, V., van Sinderen, D., Taranto, M. p … Sesma, F. (2011). B-Group vitamin production by lactic acid bacteria – current knowledge and potential applications. *Journal of Applied Microbiology, 111*(6), 1297–1309. http://doi.org/10.1111/j.1365-2672.2011.05157.x.
LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., et al. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Current Opinion in Biotechnology, 24*(2), 160–168. http://doi.org/10.1016/j.copbio.2012.08.005.
Leblanc, J., Pía Taranto, M., Molina, V., & Sesma, F. (2010). B-Group vitamins production by probiotic lactic acid bacteria. In F. Mozzi, R. R. Raya, & G. M. Vignolo (Eds.), *Biotechnology of lactic acid bacteria* (pp. 211–232). Wiley-Blackwell. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/9780813820866.ch12/summary.
Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology, 15*(2), 67–78. http://doi.org/10.1016/j.tifs.2003.09.004.
Masuda, M., Ide, M., Utsumi, H., Niiro, T., Shimamura, Y., & Murata, M. (2012). Production potency of folate, vitamin B12, and thiamine by lactic acid bacteria isolated from Japanese pickles. *Bioscience, Biotechnology, and Biochemistry, 76*(11), 2061–2067. http://doi.org/10.1271/bbb.120414.
Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín, P., & Bardowski, J. (2010). Updates in the metabolism of lactic acid bacteria. In F. Mozzi, R. R. Raya, & G. M. Vignolo (Eds.), *Biotechnology of lactic acid bacteria* (pp. 3–33). Wiley-Blackwell. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/9780813820866.ch1/summary.
Nes, I. F., Diep, D. B., & Holo, H. (2007). Bacteriocin diversity in Streptococcus and Enterococcus. *Journal of Bacteriology, 189*(4), 1189–1198. http://doi.org/10.1128/JB.01254-06.
Patel, A., & Prajapati, J. B. (2013). Food and health applications of exopolysaccharides produced by lactic acid bacteria. *Advances in Dairy Research, 01*(02). http://doi.org/10.4172/2329-888X.1000107.
Paucean, A., Vodnar, D.-C., Socaci, S.-A., & Socaciu, C. (2013). Carbohydrate metabolic conversions to lactic acid and volatile derivatives, as influenced by Lactobacillus plantarum ATCC 8014 and Lactobacillus casei ATCC 393 efficiency during in vitro and sourdough fermentation. *European Food Research and Technology, 237*(5), 679–689. http://doi.org/10.1007/s00217-013-2042-6.
Piva, A., & Headon, D. R. (1994). Pediocin A, a bacteriocin produced by Pediococcus pentosaceus FBB61. *Microbiology (Reading, England), 140*(Pt 4), 697–702.
Rossi, M., Amaretti, A., & Raimondi, S. (2011). Folate production by probiotic bacteria. *Nutrients, 3*(1), 118–134. http://doi.org/10.3390/nu3010118.
Salvucci, E., Hebert, E. M., Sesma, F., & Saavedra, L. (2010). Combined effect of synthetic enterocin CRL35 with cell wall, membrane-acting antibiotics and muranolytic enzymes against Listeria cells. *Letters in Applied Microbiology, 51*(2), 191–195.
Salvucci, E., Saavedra, L., & Sesma, F. (2007). Short peptides derived from the NH2-terminus of subclass IIa bacteriocin enterocin CRL35 show antimicrobial activity. *Journal of Antimicrobial Chemotherapy, 59*(6), 1102–1108.
Vermelho, A. B., Meirelles, M. N., Lopes, A., Petinate, S. D., Chaia, A. A., & Branquinha, M. H. (1996). Detection of extracellular proteases from microorganisms on agar plates. *Memórias Do Instituto Oswaldo Cruz, 91*(6), 755–760.
Yang, S.-C., Lin, C.-H., Sung, C. T., & Fang, J.-Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Food Microbiology, 5*(241). http://doi.org/10.3389/fmicb.2014.00241.