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Molecular identification and technological characterization of lactic acid bacteria isolated from fermented kidney beans flours (*Phaseolus vulgaris* L. and *P. coccineus*) in northwestern Argentina

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ABSTRACT

Legumes are an important protein source in developing countries and their flours represent an attractive alternative for the manufacture of gluten free products. In the present study, 4 kidney bean varieties (Alubia, Pallar, Black and Red beans) commonly cultivated in northwestern Argentina, were milled and spontaneously fermented in order to isolate and select autochthonous lactic acid bacteria (LAB) with relevant technological and functional properties for usage as starter cultures. Twelve doughs were fermented with daily back-slopping at 37 °C for 6 days and evolution of total mesophiles, lactic acid bacteria, and yeasts and molds populations were followed by plate counting. A combination of phenotypic and genotypic methods including $(GTG)_5$-based PCR fingerprinting and 16S rRNA gene sequencing were used to differentiate and identify the isolated LAB to species level. LAB counts ranged from around 0.89 ± 0.81 to 8.74 ± 0.03 log cfu/g with a pH decline from 6.4 to 3.9 throughout fermentation. Four genera and nine species of LAB: *Enterococcus durans*, *E. faecium*, *E. mundtii*, *E. casseliflavus*; *Lactobacillus rhamnosus*, *Lactococcus garvieae*, *Weissella cibaria* and *W. paramesenteroides* were found on kidney beans. Twenty five LAB strains were assessed for their abilities to grow on kidney bean extracts, acidifying capacities (pH and acidification rates), amylolytic, proteolytic, tannase and gallate decarboxylase activities as well as pathogens inhibition by antimicrobials. Based on these properties *E. durans* CRL 2178 and *W. paramesenteroides* CRL 2182 were inoculated singly and combined in Alubia kidney bean flour and fermented for 24 h at 37 °C. LAB strains were beneficial for removing trypsin inhibitors and tannins from sourdoughs and for improving amino acids and phenolics contents, increasing the antioxidant activities of kidney bean matrices. Selected strains have potential as starter cultures for obtaining fermented bean products with high nutritional and functional quality.

1. Introduction

Legumes (such as beans, chickpea, lentils and peas among other pulses belonging to the *Fabaceae* Family) are crops widely produced and consumed around the world because of their nutritional quality. They constitute an important source of dietary protein (20 to 40% of dry weight) with high biological value and represent an attractive option for diets in which the consumption of animal protein is limited by non-availability or restricted by religious or cultural habits (Boye, Zare, & Pletch, 2010). Apart from their nutritional properties, legume proteins also possess functional properties (water binding capacity, fat absorption, foaming and gelation) that play an important role in formulation and processing of food (Boye et al., 2010; Campos-Vega, Loarca-Piña, & Oomah, 2010). In addition, pulses provide complex carbohydrates, high dietary fiber levels, vitamins and minerals required for human health (Suárez-Martínez et al., 2016). Besides nutrients, some bioactive phytochemicals such as peptides and phenolic compounds with antioxidative properties have been reported for legumes (Campos-Vega et al., 2010; Moreno-Jiménez et al., 2015). Their daily consumption has been associated with many health benefits that have increased the interest in using pulses and ingredients derived from them in the development of novel foods (Boye et al., 2010; Moreno-Jiménez et al., 2015; Suárez-Martínez et al., 2016). The growing diagnostic of celiac disease patients have also increased the study of legumes as alternative to common flours for preparing gluten-free bakery products, pasta, extruded products and ready-to eat snacks (Laleg, Cassan, Barron, Prabhasankar, & Micard, 2016). In view of all their benefits, global legumes production has significantly raised in last years and the 68th United Nations General Assembly declared 2016 as the International Year of Pulses (IYP) (A/RES/68/231, FAO-WHO, 2015).

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The common bean (*Phaseolus vulgaris* L.), is one of the most important grain legumes used in human nutrition. It comprises 50% of the legumes consumed worldwide and is considered a primary source of dietary protein in developing countries (Câmara, Urrea, & Schlegel, 2013). In fact, it is a basic component of diet of large Latin American sectors. Argentina has a prominent role in the production and export of different varieties of common beans (*Phaseolus vulgaris* L., *P. coccineus* and *P. lunatus*) with their main production areas concentrated in the provinces of Salta, Jujuy and Tucuman, in the northwestern region of the country. In 2016, Argentina produced 660.000 tons of pulses (67% corresponded to different varieties of common beans) and ranked eleventh among the world's leading producers (Source: Argentine Chamber of Legumes, http://www.clera.com.ar). Beans are consumed in multiple forms such as unprocessed seeds, canned products, or as gluten-free wheat flour substitute (Câmara et al., 2013; Miñarro, Albanell, Aguilar, Guamis, & Capellaset, 2012). However, despite their nutritional and functional properties, raw beans contain many undesirable compounds considered as anti-nutritional factors (ANF), which reduce protein digestibility, nutrient absorption and are responsible for intestinal discomfort (Khokhar & Owusu Apenten, 2009). Fermentation represents an attractive alternative for processing legumes that may improve their nutritional and nutraceutical properties (Curiel et al., 2015; Gan, Shah, Wang, Lui, & Corke, 2016; Jhan, Chang, Wang, Chou, & Young, 2015; Limón et al., 2015) and may increase their digestibility by eliminating the ANF contained in the seeds. The effect of microbial fermentation on ANF was studied for various legumes (Bartkiene, Krungleviciute, Juodeikiene, Vidmantiene, & Maknickiene, 2014; Rizzello et al., 2016). The partial or complete elimination of α-galactosides, tannins, phytic acid and trypsin inhibitory activity was obtained through the spontaneous fermentation of kidney beans (Granito et al., 2002) or by the lactic fermentation of different legume flours produced by selected *Lactobacillus* strains (Coda et al., 2015; Curiel et al., 2015; Starzyńska-Janiszewska & Stodolak, 2011). In this regard, lactic acid bacteria (LAB) are the microorganisms of choice for food fermentation due to their technological properties and GRAS status. There is consensus that fermentation of different food matrices with LAB strains, such as those of *Lactobacillus* genus, can improve organoleptic properties (flavor and texture) and safety (by antimicrobials production) of the product and may release bioactive metabolites, providing health benefits beyond basic nutrition (Das & Goyal, 2012). Fermentation process can rely on the natural microbiota present in raw materials and the environment or be controlled by inoculation with commercial starters. It is well-known that best results are obtained with autochthonous rather than allochthonous starters (Di Cagno, Coda, De Angelis, & Gobbetti, 2013). Unfortunately, most commercially available strains are allochthonous for fermentation of different raw vegetables being not competitive with endogenous microbiota. Then, isolation and screening of LAB from spontaneous fermentation of the food matrix of interest, could be a powerful mean of obtaining useful autochthonous cultures for commercial purposes.

In Argentina, no studies have addressed and characterized the lactic microbiota of common beans, neither their potential applications to the development of functional foods. Therefore, the present study focused on a) the isolation and molecular identification of the autochthonous LAB microbiota present in different varieties of kidney beans commonly cultivated and consumed in northwestern Argentina, and b) the technological characterization of the microorganisms in order to select the most suitable strains for developing legume fermented products. Results derived from this work will provide valuable information on the potential application of LAB to the formulation of added-value kidney bean based products with impact on regional economy.

2. Materials and methods

2.1. Preparation and sampling of sourdoughs

Twelve samples of different varieties of common beans [*Phaseolus vulgaris* L. var. *Alubia* (5), *Red* (2), *Black* (2) and *P. coccineus* var. *Pallar* (3)], were obtained from local markets. Samples were milled using Thermomix (Vorwerk, Madrid, Spain) and flours obtained were used for laboratory sourdoughs production. Doughs were prepared by mixing 50 g of flour and 50 mL of sterile water in sterile flask, with a resulting dough yield [(dough mass / flour mass) × 100] of 200 followed by an incubation of 24 h at 37 °C. Five back slopping renewals were daily performed by inoculating a fresh water-flour mixture (95 mL of water and 95 g of bean flour) with 10 g of ripe sourdough followed by the same incubation conditions as above. Samples of sourdoughs from 0 (dough, D0), 1, 3 and 5 back-slopping cycles (BS1, BS3 and BS5) were taken for acidity and microbiological analysis. The pH-values were determined with a pH meter Altronix TPX I (NY, USA) and total titratable acidity (TTA) according to AACC (2000) method. Two sourdoughs series for each bean variety were obtained by independent propagations and analyzed.

2.2. Microbiological analysis and lactic acid bacteria isolation

Ten grams of each sourdough was homogenized with 90 mL of sterile 0.85% (w/v) NaCl in a lab paddle blender (Stomacher® 400, Seward, Worthing, UK) during 3 min, ten-fold diluted in this solution and spread on selective agar media for isolation and count of different microorganisms. The following culture media and incubation conditions were used: MRS (de Man, Rogosa & Sharpe; Britania, Argentina) supplemented with 0.1% cycloheximide 2000 U (Sigma, St. Louis), 48 h at 37 °C under microaerophilic conditions, Rogosa agar (Oxoid), 72 h in anaerobiosis (AnaeroJar Oxoid, UK), Plate Count Agar (Britania), 24 h at 30 °C in aerobiosis and Yeast and Mold Growth Medium (Britania), 5 days at 30 °C in aerobiosis. Means and standard deviations of plates containing 100–300 cfu were calculated, and at least 10 colonies grown in MRS and Rogosa agar with different morphologies were transferred to MRS broth and stored at − 70 °C in skim milk supplemented with 10% glycerol.

2.3. Genotypic identification of the isolates

2.3.1. Chromosomal DNA extraction

Five milliliters of a stationary phase culture grown in MRS, were used for DNA extraction according to Pospiech and Neumann (1995) with minor modifications. Cells were washed with SET buffer (20 mM Tris-HCl, 75 mM NaCl, 25 mM EDTA; pH 7.5) and resuspended in SET buffer containing 15 mg/mL lysozyme (Sigma, St. Louis). After 90 min incubation at 37 °C, 1/10 vol of 10% SDS and 20 mg/mL of proteinase K were added and mixtures were incubated at 55 °C for 2 h. Then, 1/3 vol of 5 M NaCl and 1 vol of chloroform-methanol (24:1) were added allowing to stand at room temperature for 30 min. The aqueous phase obtained by centrifugation (10,500 rpm, 10 min) was transferred to another tube and DNA was recovered by precipitation with isopropanol (1:1 v/v). Precipitated DNA was washed with 70% ethanol (10 min at 10,500 rpm), dried by alcohol evaporation and resuspended in milliQ water with RNAse (0.1 mg/mL) DNAse free (30 min at 37 °C).

2.3.2. Rep-PCR: repetitive element (or extragenic) palindromic-Polymerase Chain Reaction fingerprinting method

The rep-PCR reactions were performed in a thermocycler (MyCicler, BIORAD, Hercules, USA) using the primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′). PCR mixtures contained: 4 μL of 5 × buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]; 2 μL of a mixture of deoxyribonucleoside triphosphates (dATP, dTTP, dCTP and dGTP, 5 mM); 1 μL GoTaq polymerase (Promega, Madison, USA); 2 μL of primer

(10 mM); 1 μL of purified chromosomal DNA (100 ng/μL) as template and 15 μL milliQ water. PCR conditions were: preliminary denaturation 5 min at 95 °C; amplification in 30 cycles: denaturation 94 °C for 1 min, annealing 1 min at 40 °C, extension 8 min at 65 °C and final extension at 65 °C for 16 min. Electrophoresis was carried out in agarose 1.5% (w/v) gel at a constant voltage of 40 V for 4 h in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). For DNA visualization, gels were stained with the dye GelRed (Biotium, USA) visualized using a transilluminator (Syngene, UK). Rep-PCR fingerprints were analyzed using the software Bionumerics 7.50 (Applied Maths, Belgium) and dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

2.3.3. 16S ribosomal RNA gene sequencing analysis

One representative of each rep-PCR fingerprint group was subjected to 16S rRNA gene sequencing. For identification of the isolates, V1 variable region of the gene coding for 16S ribosomal RNA were amplified using PLB16 (5′ AGA GTT TGA TCC TGG CTC AG 3′) and MLB16 (5′ GGC CAC TGC TGG GTA GTT AG 3′) primers. The PCR mixture contained 10 μL of 5 × buffer [20 mM Tris-HCl (pH 8.4), 500 mM KCl]; 2 μL of a mixture of deoxyribonucleoside triphosphates (dATP, dTTP, dCTP and dGTP, 5 mM); 0.4 μL of GoTaq polymerase; 10 μL of each primer (10 mM); 2 μL of purified chromosomal DNA (100 ng/μL) as template and enough milliQ water to bring the volume to 50 μL. The amplification program consisted of 5 min of denaturation at 94 °C, followed by 30 cycles of 94 °C (30 s), 52 °C (30 s) and 72 °C (45 s); and an elongation step of 10 min at 72 °C. PCR products were electrophoresed in 1% (w/v) agarose gels, stained and visualized as described above. Amplicons were purified and sequenced at CERELA-CONICET by using an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA, USA). 16S rRNA gene sequence alignments were performed using the multiple sequence alignment method and identification queries were fulfilled by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and in the Ribosomal Database Project (https://rdp.cme.msu.edu/). The partial 16S rRNA gene sequences of LAB were submitted to the European Nucleotide Archive database (LT714198–LT714200 and LT900369–LT900390).

2.4. Technological evaluation of the isolates

2.4.1. Acidification capacity

The kinetic of acidification of the isolates was evaluated in a sterile flour extract (SFE) liquid broth proposed by Alfonzo et al. (2013) with some modifications. *Phaseolus vulgaris* var. Alubia flour with the following proximate composition (%): moisture, 9.4; protein 32.3; fat, 1.04; ash, 5.6; fiber, 1.7; carbohydrates, 51.0; was suspended at 40% (w/v) in distilled H_2O, stirred at 4 °C during 2 h, and then sterilized at 121 °C for 20 min. Supernatant obtained by centrifugation (8000 rpm, 10 min, 4 °C) was used as liquid medium. LAB isolates grown in MRS broth at 37 °C for 16 h were washed twice (8000 rpm, 10 min) with sterile 0.85% (w/v) NaCl and re-suspended to a final OD_{560} of 0.8 in the same solution. Cell suspensions were inoculated (2%) in SFE and incubated at 37 °C for 24 h. Samples for pH and proteolytic activity determinations were taken at 0, 3, 6, 9 and 24 h.

2.4.2. Proteolytic activity

The ability of LAB isolates to release amino acids from proteins was determined spectrophotometrically by the *o*-phthaldialdehyde (OPA) procedure of Church, Swaisgood, Porter, and Catignani (1983). Samples were deproteinized with 0.75 M trichloroacetic acid and the supernatants were incubated with the o-PA solution (50 mL sodium tetraborate 100 mM, 5 mL sodium dodecyl sulfate 20% (w/v), 2 mL o-Pa 40 mg/mL dissolved in methanol, 0.2 mL β-mercaptoethanol) for 10 min at room temperature before reading their OD_{340}. The results were expressed as μmol/mL of leucine released by referring to a standard curve of L-leucine.

2.4.3. Amylolytic activity

The ability of isolates to hydrolyze starch in agar was assessed as amylolytic activity. Active LAB were streaked on plates containing MRS agar with 1% starch instead glucose that were incubated at 37 °C for 48 h. Grown plates were stored at 4 °C during 24 h and then flooded with 4% (w/v) iodine solution. A clear zone around the colonies was indicative of amylase production and compared with *Lactobacillus amylolyticus* CRL 1949 used as positive control.

2.5. Tannase activity

Cultures grown on MRS agar plates were harvested with sterile cotton swabs and suspended in 1 mL of substrate medium (pH 5.0) containing NaH_2PO_4 (33 mmol/L) and methyl gallate (20 mmol/L) in order to obtain bacterial suspensions of OD_{560}:1. Suspensions were incubated aerobically at 37 °C for 24 h and then alkalinized with an equal amount of 2 N $NaHCO_3$ solution (pH 8.6). After exposure to air for 1 h at room temperature (25 °C), the development of a green to brown color of the medium was considered positive for tannase activity (Osawa, Kuroiso, Goto, & Shimizu, 2000).

2.6. Gallate decarboxylase activity

Overnight cultures in MRS broth were inoculated at 1% (v/v) in 10 ml of MRS broth containing 10 mM gallic acid and incubated anaerobically at 37 °C for 3 days. After incubation, the cultures were alkalinized with equal amounts of 2 N $NaHCO_3$ solution (pH 8.6) and incubated aerobically at 37 °C for 1 h. Development of light yellow to brown color of the medium was taken as positive for gallate decarboxylase activity (Osawa et al., 2000).

2.7. Inhibition of pathogens

The ability of the isolates to produce antimicrobial substances was determined by an agar well diffusion assay. Three common food pathogens, *Bacillus cereus* MBC2 (from INIQUI-CONICET), *Escherichia coli* C3 (from Institute of Microbiology “Luis Verna” of University of Tucumán) and *Listeria innocua* 7 (from Unité de Recherches Laitieres et Genetique Appliqué, INRA, France) were used as indicator strains. Overnight cultures of LAB in MRS at 37 °C were harvested by centrifugation (6000 rpm, 10 min at 4 °C) and filtered through 0.22-μm pore-size filters to obtain cell-free supernatants (CFS). To elucidate the nature of antimicrobial compounds, aliquots of CFS were adjusted to pH 6.5 with 1 N NaOH, added with catalase (300 IU/mL) or proteinase K (1 mg/mL, 3 h at 37 °C) in order to discriminate acid, H_2O_2 or bacteriocin inhibition. Untreated and treated CFS were inoculated (50 μL) in wells made in BHI agar plates seeded with each pathogen and incubated at 37 °C for 24 h. The appearance of inhibition halos around each well was taken as positive result for antimicrobials production.

2.8. Fermentation of kidney beans flour with selected strains

Selected sourdough lactic acid bacteria, *Weissella paramesenteroides* CRL 2181 and *Enterococcus durans* CRL 2178 were grown separately on MRS broth at 37 °C until late exponential phase, washed twice (10,000 rpm, 10 min) in 0.9% NaCl and resuspended in tap water at 8.0 log cfu/mL. *Phaseolus vulgaris* var. Alubia flour was mixed with each bacterial suspension and a mixture of strains (1:1 ratio) in order to obtain an initial concentration of about 7.0 log cfu/g of dough. Doughs (with DY160 corresponding to 62.5 and 37.5% of flour and water, respectively) were homogenized and incubated at 37 °C for 24 h. Control dough without bacterial inoculum was prepared and incubated under the same conditions.

2.9. Microbiological counts and technological-functional properties of fermented doughs

Microbiological counts, pH, TTA and FAA were determined as described in Sections 2.2 and 2.4.

2.9.1. Trypsin inhibitors activity

Trypsin inhibitors activity (TIA) was determined according to Kakade, Simons, and Liener (1969) and Smith, Megen, Twaalfhoven, and Hitchcock (1980). Sourdoughs extracts were prepared with 1 g of flour and 50 mL of 0.01 M NaOH and kept on a magnetic stirrer for 3 h. The synthetic substrate BAPNA (*N*-benzoyl-DL-arginine *p*-nitroanilide, 0.4 g/L in 0.05 M Tris-buffer, pH 8.2) was subjected to hydrolysis by Trypsin (type III, from bovine pancreas, 20 mg/L in HCl 0,001 M) to produce yellow-colored *p*-nitroanilide. The degree of inhibition of yellow-color production by the sourdoughs extracts in 10 min at 37 °C was measured at 410 nm using a spectrophotometer (Versamax, Molecular Devices, California, USA). TIA was expressed as milligrams of trypsin inhibited/g sample (mg/g).

2.9.2. Total polyphenols and tannins

Total phenolic contents and tannins were determined according to Makkar, Blummel, Borowy, and Becker (1993). Reaction of samples with 1 N Folin-Ciocalteu reagent and Polyvinylpyrrolidone (PVPP, Sigma) followed by absorbances reading at 725 nm were used to assess total polyphenols and non-tannin phenolic compounds, respectively. Tannin contents were calculated as the difference between total phenolics and non-tannin phenolics and expressed as gallic acid equivalents per gram of sample (GAE/g).

2.9.3. Antioxidant activity

DPPH radical scavenging capacity of sourdoughs extracts was determined according to Hung, Maeda, Miyatake, and Morita (2009). Briefly, 0.1 mL of extract was mixed with 3.9 mL of methanolic DPPH solution (6×10^{-5} mol/L), vigorously shaken and allowed to stand at room temperature in the dark for 20 min. The decrease in absorbance of the resulting solution was determined at 515 nm. Blank was prepared using 3.9 mL DPPH and 0.1 mL methanol and its absorbance was measured at t = 0. The antioxidant activity was calculated as:

$$\%\text{DPPH scavenging} = (1 - \text{Abs sample}_{t20}/\text{Abs control}_{t0}) \times 100$$

2.10. Statistical analysis

The results are expressed as the average of two independent trials ± standard deviation for all sourdoughs analysis and three assays ± SD for characterization of LAB isolates. Significant differences between means were determined by Tukey's test after analysis of variance (one-way ANOVA) with Minitab Statistic Program, release 12 for Windows. A P value of < 0.05 was considered statistically significant. Technological properties of strains were analyzed through Principal Component Analysis using the software XLSTAT (19.4).

3. Results and discussion

3.1. Microbiological, pH and acidity analysis

Kidney bean is a prominent legume crop in the North-western region of Argentina with potential application to the development of novel functional and gluten-free foods. In the present study, unfermented and spontaneous fermented flours of 4 common bean varieties after five back-slopping steps (sourdoughs), were microbiologically analyzed in the search of autochthonous LAB that could be used in the future as starter cultures for functional legume derived products. The changes in the viable counts of most relevant microbial populations, pH and titratable acidity of the sourdoughs during six days of fermentation are presented in Fig. 1 and Table 1, respectively. Sourdoughs fermentation was characterized by the increase in LAB number from the first day of fermentation reaching the highest values after 5 back-slopping steps in all beans varieties assayed. Initial LAB populations raised from median values of 0.89 ± 0.81; 1.43 ± 0.85; 1.59 ± 0.38 and 2.11 ± 0.11 log cfu/g of unfermented flours (doughs) to 8.63 ± 0.19; 8.69 ± 0.47; 8.03 ± 0.63 and 8.74 ± 0.03 log cfu/g at 6th day of fermentation for Pallar, Alubia, Red and Black beans sourdoughs, respectively. Other studies have reported maximal LAB values of 4 log cfu/g for wheat flours from Italian bakeries (Alfonzo et al., 2013), whereas LAB levels found in our legume flours were in agreement with those present in flours used in gluten free bread making (amaranth, chickpea, rice, corn, quinoa, and potato) for celiac people, which showed a maximum of 2.06 log cfu/g (Corsetti et al., 2007) of LAB. Fungal counts increased at the beginning of fermentations and peaked at 24 h in almost all samples, but progressively decreased in the successive back-slopped sourdoughs remaining at levels of around 3 log cfu/g (Fig. 1). It has been reported that yeasts population remain at low levels and do not exceed 5 log cfu/g in mature wheat sourdoughs incubated at 37 °C (Vrancken, Rimaux, Weckx, Leroy, & De Vuyst, 2011). Dominance of microbial types and species found in mature sourdoughs may be influenced by temperature and flour characteristics (Minervini, De Angelis, Di Cagno, & Gobbetti, 2014). The obtained mean counts for total mesophiles showed some differences among bean varieties ranging from 3.65 ± 0.13 and 4.53 ± 0.13 for unfermented Black and Red bean doughs to 9.71 ± 0.39 and 8.70 ± 0.15 log cfu/g after 6 days of sourdoughs propagation, respectively. Our microbiological data were comparable to the obtained by Curiel et al. (2015) who assessed microbial populations of traditional Italian legumes doughs and LAB in sourdoughs fermented with *L. plantarum* C48 and *L. brevis* AM7. These authors reported that total mesophilic aerobic bacteria ranged from 1.6 to 4.3 log cfu/g whereas the number of presumptive lactic acid bacteria varied from 1.0 to 2.5 log cfu/g, and yeasts and molds were found at 1.1–3.5 log cfu/g for most of the flours. When selected lactobacilli strains were used as starters for sourdough fermentation, the cell density of presumptive LAB reached values of 9.8–10.2 log cfu/g (Curiel et al., 2015). As expected, the progressive dominance of LAB in our study, paralleled the decrease in pH and the increase of titratable acidities of sourdoughs: pHs decreased from an initial value of around 6.30 ± 0.14 of unfermented doughs to about 3.95 ± 0.07 after 6 days of propagation using the back-slopping procedure, whereas acidities raised from around 4.23 ± 0.06 to 23.45 ± 0.21 mL of 0.1 M NaOH per 10 g of sourdough. Slight differences were found among bean varieties at BS3 and BS5 (Table 1). Italian legume sourdoughs LAB fermented, reached pHs (4.0 to 4.4) and TTA values (20.4 to 27.0 mL NaOH/10 g of dough) (Curiel et al., 2015) similar to the produced by the wild LAB microbiota present in our beans sourdoughs after 5 back-slopping steps.

Sourdough fermentation is an old and traditional food technology recognized for its positive effects on the sensory, structural, nutritional properties and shelf life of bakery products (Gänzle & Ripari, 2016). It has been reported that microbiota of stable sourdoughs of cereals and pseudocereals worldwide, principally consists of LAB and yeasts (Coda, Di Cagno, Gobbetti, & Rizzello, 2014; Corsetti & Settanni, 2007; De Vuyst et al., 2014; Huys, Daniel, & De Vuyst, 2013). However, information about legume sourdoughs is still scarce. In a recent study, Rizzello, Calasso, Campanella, De Angelis, and Gobbetti (2014) prepared and propagated wheat-legume (chickpea, lentil and bean) sourdoughs for the manufacture of Italian breads and found out that this mixed sourdough showed a higher number of presumptive LAB, as well as faster and higher acidification compared to cereals sourdoughs. Different studies also revealed that daily back-slopped sourdoughs of cereals and cereals-legumes stabilized in LAB consortium and acidification parameters within 3 to 10 days (Rizzello et al., 2014; Van der Meulen et al., 2007; Vrancken et al., 2011).

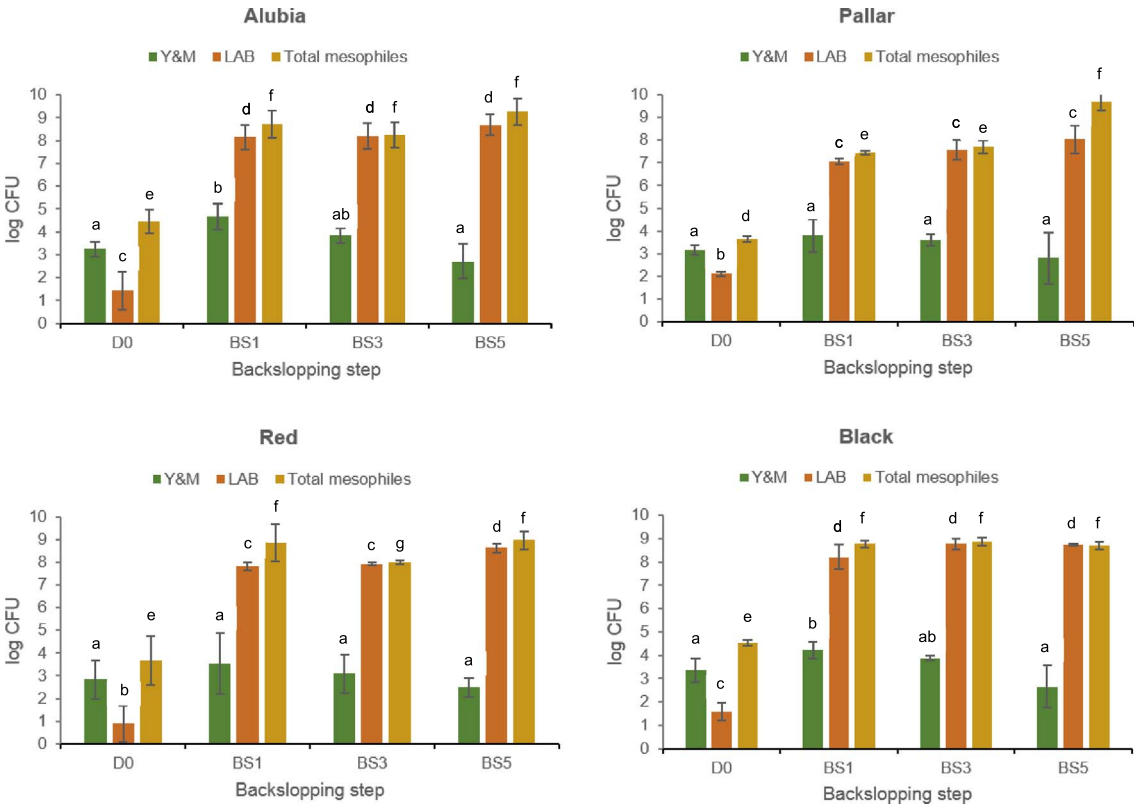

Fig. 1. Microbiological analyses of the Argentinian legume flours before (doughs) and after 5 back-slopping steps (sourdoughs) incubated for 24 h at 37 °C. Total mesophilic aerobic bacteria were estimated on Plate Count Agar (PCA), lactic acid bacteria on MRS agar; yeasts and molds on Yeasts and Molds selective Agar. The data are the means of three determinations ± standard deviations of two independent samples. Each microbial group at different backslopping steps was compared with its respective dough (BS0). Bars with different superscript letters (a, b, c, d, e and f) differ significantly ($P < 0.05$).

Table 1
pH and tritatable acidity of beans sourdoughs at each back-slopping step. Standard deviations are shown with (±). Means within each column with different letters are significantly different ($P \leq 0.05$).

Bean variety	pH				TTA (mL of 0.1 M NaOH/10 g)			
	BS_0*	BS_1	BS_3	BS_5	BS_0	BS_1	BS_3	BS_5
Alubia	6.33 ± 0.08^a	4.82 ± 0.08^a	4.48 ± 0.04^a	4.14 ± 0.19^a	4.40 ± 0.14^a	19.44 ± 0.59^a	21.32 ± 0.26^a	22.18 ± 0.58^a
Pallar	6.43 ± 0.06^a	4.80 ± 0.10^a	4.57 ± 0.04^b	4.20 ± 0.10^a	4.23 ± 0.06^b	19.70 ± 1.08^a	20.80 ± 0.56^a	22.17 ± 0.67^a
Black	6.35 ± 0.07^a	4.80 ± 0.00^a	4.35 ± 0.07^c	4.00 ± 0.14^a	4.35 ± 0.07^a	19.65 ± 0.35^a	21.60 ± 0.14^b	23.45 ± 0.21^b
Red	6.30 ± 0.14^a	4.80 ± 0.14^a	4.30 ± 0.00^c	3.95 ± 0.07^a	4.40 ± 0.14^a	19.65 ± 1.77^a	21.90 ± 0.14^c	23.05 ± 0.07^c

* Back-slopping cycle.

3.2. Strain typing by rep-PCR

A total of 83 colonies were picked from MRS and Rogosa agar plates, whereas 63 isolates (22 isolates from Alubia, 18 from Pallar, 12 from Black and 11 from Red beans) were selected as presumptive LAB based on cell morphology (non-motile bacilli and cocci), Gram staining (Gram positive) and catalase (negative) results. All isolates were subjected to rep-PCR $(GTG)_5$ fingerprinting technique for genotypic grouping. Amplification of repetitive regions of bacterial genomes is considered a fast and reliable method, with high differentiation power for taxonomy, molecular genotyping, and determination of phylogenetic relationships between closely related species and even distinct bacterial strains (Tafvizi & Tajabadi-Ebrahimi, 2015). Analysis of the $(GTG)_5$-PCR fingerprint band patterns obtained is shown in Fig. 2. The isolates were grouped in 10 clusters at a similarity level of 70%. As seen in the dendrogram obtained by UPGMA algorithm, the first 3 clusters presented great variability of band patterns that resulted in 12 distinct profiles. The highest diversity of genera and species were then identified within these clusters which also contained the highest heterogeneity of microorganisms according to their origin and day of isolation (Table 2). Clusters IV to VII were more homogenous in band profiles, species isolated and their origin. It must be highlighted that clusters V and VI contained isolates coming from the first back-slopping step of different samples, suggesting adaptation and proliferation under similar environmental conditions. The remaining clusters (VII–X) contained isolates that shared origin and displayed a high similarity between band patterns so that only one profile from each one was further sequenced. As global result, 25 distinct rep-PCR profiles were identified by the UPGMA algorithm, and one representative of each of them was subjected to sequencing of 16S rRNA gene in order to obtain the identities of the strains and confirm their inclusion within each cluster. Partial 16S rRNA gene sequences obtained were compared with database at NCBI and RDP revealing high similarity values to a number of sequences in both database. The isolates were identified as *Lactobacillus rhamnosus* (1), *Lactococcus garvieae* (3 strains), *Weissella* spp. (9 isolates: 5 *W. cibaria* and 4 *W. paramesenteroides*) and *Enterococcus* spp. (12 isolates: 4 *E. durans*, 5 *E. faecium*, 2 *E. casseliflavus* and 1 *E. mundtii*). These strains were deposited at CERELA Culture Collection and a CRL number was assigned (Table 2). The sequence of the partial 16S rRNA gene of LAB was submitted to the European

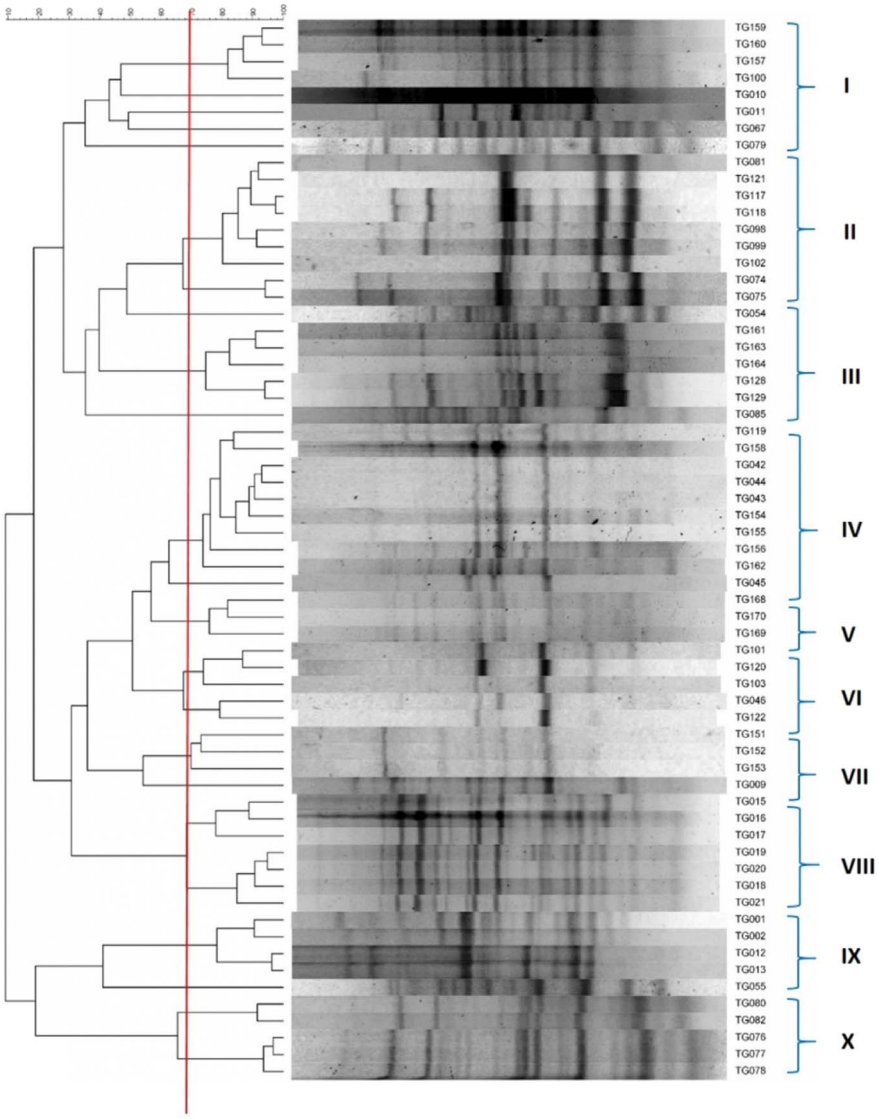

Fig. 2. Dendrogram obtained by cluster analysis of rep-PCR (GTG_5) fingerprints. The dendrogram is based on Dices's Coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA). Representative isolates were identified by 16S rRNA gene sequencing.

Nucleotide Archive database (accession numbers LT714198–LT714200 and LT900369–LT900390). Table 3 shows the presence of the different species identified in each bean variety. Only the genus *Enterococcus* was present in the four bean varieties analyzed although no species was presents in all of them. *Weissella* species were present in Alubia, Pallar and Black beans, whereas *Lactococcus garvieae* was isolated from Red and Black beans and *Lactobacillus rhamnosus* was detected only in Red beans. Both Alubia and Pallar varieties contained four LAB species whereas Red and Black beans contained only three. At the last sourdough stage analyzed, LAB microbiota included *Lactobacillus rhamnosus, E. casseliflavus*, *Weissella cibaria* and *W. paramesenteroides* (Table 2). Many studies have investigated the composition of the microbiota of traditional daily backslopped sourdoughs, with both culture-dependent and culture-independent techniques (Corsetti & Settanni, 2007; De Vuyst et al., 2014; Minervini et al., 2012). A wide range of LAB have been isolated from mature sourdoughs made in different countries (> 80 species) and although most of them belong to the genus *Lactobacillus*, other genera such as *Leuconostoc*, *Weissella*, *Pediococcus* and *Enterococcus* have also been identified (Alfonzo et al., 2013; Corsetti & Settanni, 2007; De Vuyst et al., 2014; Huys et al., 2013). Among them, *Lactobacillus sanfranciscensis*, *L. plantarum*, *L. brevis* and *L. paralimentarius* are the LAB species most frequently found during fermentation of cereals and pseudocereals sourdoughs such as wheat, maize, sorghum, rye and quinoa (Coda et al., 2014; De Vuyst et al., 2014; Gänzle & Ripari, 2016; Minervini et al., 2012; Ruiz Rodríguez et al., 2016). However, it has been stated that sourdough microbial diversity and stability may be affected by ecological factors that include specific technological parameters (e.g., percentage of inoculum, time and temperature of fermentation), those uncontrolled by the operator (e.g., chemical, enzyme and microbial composition of flour); microbial adaptability and metabolic activities, interactions between microorganisms, among others (Gänzle & Ripari, 2016; Minervini et al., 2014). In our research, the beans analyzed contained less LAB diversity than the reported by other studies with a total of 4 genera and 9 species and no > 4 species per bean variety. It has been reported that α-diversity of a single sourdough is limited; with < 6 different species or strains accounting for > 99% of microbial cells (Gänzle & Ripari, 2016). In the present study, *Lactobacillus rhamnosus* was the only lactobacillus detected and, as far as we know, it is the first report of this species in legume-sourdoughs. *L. rhamnosus* has only been reported previously in spontaneous sorghum flour fermentations (Madoroba et al., 2009). However, more studies are needed in order to compare results and make definite conclusions about the microbial diversity in beans.

3.3. Technological properties of LAB bean isolates

Appropriate processing is probably more important for legumes than for any other food group, due to their high ANF contents. Traditional methods of processing in developing countries include soaking, cooking, sprouting, and fermentation. Fermentation is an

Table 2
Molecular identification by 16S rDNA sequence analysis of LAB isolated from beans sourdoughs fermentation.

Isolate	CRL no	Bean variety	Backslopping day	Dendrogram cluster	Closest relative	% identity	Accession no
TG1	2147	Alubia	1	IX	*Weissella paramesenteroides*	99%	LT900369
TG9	2165	Alubia	1	VII	*Enterococcus durans*	100%	LT900370
TG10	2148	Alubia	3	I	*Weissella cibaria*	99%	LT900371
TG12	2149	Alubia	3	IX	*Weissella paramesenteroides*	99%	LT900372
TG15	2150	Pallar	1	VIII	*Enterococcus faecium*	99%	LT900373
TG42	2166	Alubia	1	IV	*Enterococcus durans*	100%	LT900374
TG55	2180	Red	5	IX	*Weissella cibaria*	98%	LT900375
TG67	2167	Red	5	I	*Lactobacillus rhamnosus*	100%	LT714199
TG74	2170	Red	3	II	*Lactococcus garvieae*	100%	LT900376
TG76	2168	Red	5	X	*Enterococcus casseliflavus*	100%	LT900377
TG81	2169	Pallar	5	II	*Enterococcus casseliflavus*	99%	LT900378
TG98	2172	Black	1	II	*Lactococcus garvieae*	99%	LT900379
TG100	2171	Black	3	I	*Weissella cibaria*	99%	LT900380
TG101	2173	Black	1	VI	*Enterococcus faecium*	100%	LT900381
TG102	2174	Black	3	II	*Lactococcus garvieae*	100%	LT900382
TG117	2175	Black	1	II	*Enterococcus faecium*	100%	LT900383
TG119	2176	Pallar	1	IV	*Enterococcus faecium*	99%	LT900384
TG120	2181	Pallar	1	VI	*Weissella paramesenteroides*	99%	LT900385
TG121	2182	Pallar	5	II	*Weissella paramesenteroides*	99%	LT900386
TG128	2185	Alubia	3	III	*Enterococcus mundtii*	100%	LT714200
TG151	2184	Alubia	3	VII	*Weissella cibaria*	95%	LT900387
TG158	2183	Pallar	5	IV	*Enterococcus durans*	100%	LT900388
TG160	2177	Alubia	5	I	*Weissella paramesenteroides*	99%	LT900389
TG164	2178	Alubia	3	III	*Enterococcus durans*	100%	LT714198
TG168	2179	Black	1	V	*Enterococcus faecium*	99%	LT900390

Table 3
LAB species identified in each kidney bean variety analyzed.

	Enterococcus casseliflavus	*Enterococcus durans*	*Enterococcus faecium*	*Enterococcus mundtii*	*Lactobacillus rhamnosus*	*Lactococcus garvieae*	*Weissella cibaria*	*Weissella paramesenteroides*
Alubia*		■		■			■	■
Pallar		■	■				■	■
Red	■				■	■		
Black			■			■	■	

ancient, simple and inexpensive method for enhancing the shelf life, hygienic status, nutritional and organoleptic quality of food. When selecting LAB for food fermentations, different technological properties that could be relevant for the final product have to be considered such as acidifying, proteolytic, amylolytic and lipolytic activities that contribute to preservation, flavor, texture and the nutritional characteristics of the product (Gänzle, 2014).

Growth, acidification and proteolytic activities of the 25 LAB strains previously identified, were evaluated in a sterile bean flour liquid extract (SFE) used as culture media. All strains were able to grow in this medium as was evidenced by the increase in biomass determined at intervals (3, 6, 9 and 24 h) by turbidimetry (OD_{560}). The increase in cell densities correlated with the acidification kinetics with all strains decreasing pHs to values ranging from 4.07–4.96 after 24 h of incubation (Table S1). None of the strains assayed in the present study showed amylolytic whereas proteolytic activities varied among isolates: 2 isolates showed high proteolytic activities (> 3 mmol Leu/L as was quantified by the o-PA method) whereas 16 strains displayed intermediate (1 to 3 mmol Leu/L) or low activities (< 1 mmol Leu/L). Since pulses possess a high protein content, proteolytic activity becomes relevant for LAB development and for the release of bioactive peptides and essential amino acids that may act as flavor precursors or taste-active compounds. *Enterococcus durans* CRL 2178, and *E. casseliflavus* CRL 2169 showed the highest proteolytic activities (Table S1, Supplementary data). Recently, Rizzello et al. (2015) found out that fermentation of Italian legumes (*Phaseolus vulgaris*, *Cicer arietinum*, *Lathyrus sativus*, *Lens culinaris* and *Pisum sativum*) sourdoughs with selected *L. plantarum* C48 and *L. brevis* AM7 expressing different peptidase activities, released by proteolysis of the native proteins, nine lunasin-like polypeptides with a marked inhibitory effect on the proliferation of human adenocarcinoma Caco-2.

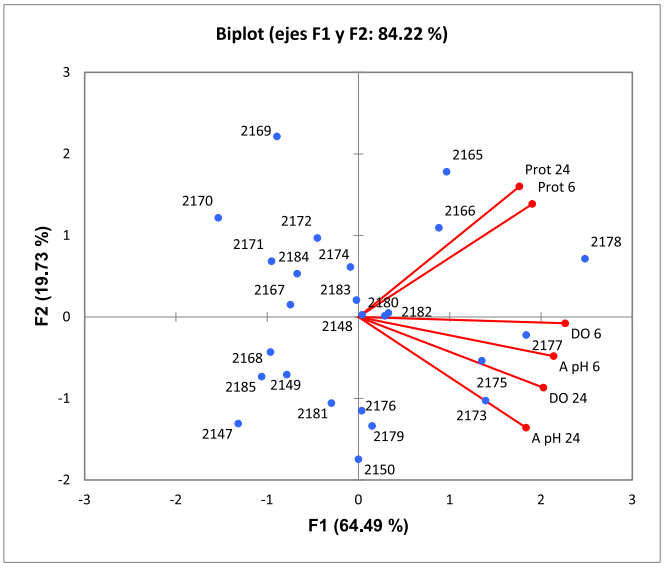

Fig. 3. Biplot graph of Principal Component Analysis (PCA) based on biomass, acidification and proteolytic activities of LAB isolated from beans sourdoughs at exponential (6 h) and stationary growth phases (24 h).

A Principal Component Analysis (PCA) based on biomass increase, acidification and proteolytic activities at exponential and stationary phases of growth of all strains was performed in order to select those with the best properties for the manufacture of fermented legumes. Analysis of PCA revealed two eigenvalues higher than 1 and the first 3 principal components (PCs) explaining 92.11% of total variation, with PC1 and PC2 accounting for 64.49% and 19.72% respectively. Fig. 3 represents the variables and observations projection in the space of PC1 and PC2. The study of the contribution of variables to factors showed

that Factor 1 was mainly related to growth and acidification whereas Factor 2 was related to proteolytic activities. The homogenous dispersion of the isolates over the four quadrants evidenced a wide variability in the parameters analyzed and the heterogeneity of LAB associated with these beans sourdoughs. The biplot graph allowed to distinguish 5 LAB strains: *Enterococcus durans* CRL 2165, 2166, and 2178; *Weissella cibaria* CRL 2180 and *W. paramesenteroides* CRL 2182 (Table S1) that present interesting technological properties (high growth and acidification in short time as well as intermediate or high proteolytic activities). In this respect, *Weissella* species have been proposed as suitable starter cultures for wheat and sorghum sourdoughs due to their ability to synthesize gluco-oligosaccharides and EPS that may improve the rheology and quality of conventional and gluten-free breads (Galle, Schwab, Arendt, & Gänzle, 2010).

3.4. Tannase and gallate decarboxylase activities

Fermentation has also been applied to the production and extraction of bioactive metabolites in foods. Then, in addition to enzyme activities related to the nutritional and organoleptic properties of fermented foods, the presence of enzymes involved in the removal of ANF and/or the generation of bioactive metabolites in the food matrix are desired features for the selection of strains as starter cultures.

Tannins are widespread in the vegetal kingdom and present in high concentrations in many plants used as food and farm feed. They are considered anti-nutritional factors (ANF) since they cause inactivation of digestive enzymes, protein insolubility, and affect the utilization of vitamins and minerals. Then, it is not advisable to ingest large quantities of tannins, as they may cause adverse health nutritional effects as well as hepatotoxicity and cancer development. Tannase (E.C. 3.1.1.20) catalyzes the hydrolysis of ester bonds in hydrolyzable tannins releasing glucose and gallic acid whereas gallate decarboxylase (E.C. 4.1.1.59) decarboxylate gallic acid to pyrogallol and CO_2. Both gallic acid and pyrogallol are considered bioactive phenolic compounds (Muñoz et al., 2017). Then, tannase and gallate decarboxylase could be relevant for removal of tannins and the release of phenolic compounds. None of the strains assayed in the present study showed tannase activity whereas 9 out of 25 strains (belonging to *L. rhamnosus*, *E. durans* and *E. faecium*, *W. cibaria* and *W. paramesenteroides* species) displayed gallate decarboxylase activity (Table 4). In the last few years, fermentation has been performed to increase the content of bioactive phenolic compounds in legumes, thus enhancing their antioxidant activity (Gan et al., 2016; Lee, Hung, & Chou, 2008; Limón et al., 2015). Metabolism of tannins has been reported previously for strains of *Lactobacillus plantarum* from different origin (Rodríguez, de las Rivas, Gómez-Cordovés, & Muñoz, 2007; Vaquero, Marcobal, & Muñoz, 2004) and for other LAB species such as *L. pentosus* and *L. paraplantarum* (Osawa et al., 2000). To our knowledge there are no reports for gallate decarboxylase activity in *Weissella* species neither for *Enterococcus durans* and *Lactobacillus rhamnosus*.

3.5. Antimicrobial activity of LAB from beans sourdoughs

Uncontrolled spontaneous vegetable fermentations and baked goods are prone to contamination with spoilage and pathogenic microorganisms which may represent economic losses and a public health hazard. Some of the common foodborne pathogens reported include *Bacillus* species, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Staphylococcus aureus* (Reij & Den Aantrekker, 2004). Conventional methods used to counter undesirable microbiota are based on chemical additives (propionic, sorbic or benzoic acids) which may affect the growth of the starter culture. Therefore, biopreservation by the addition of antibacterial LAB have been considered as a safe strategy to overcome microbial food contamination during food processing. In the present study, the ability of the 25 isolated LAB to inhibit growth of *Escherichia coli*, *Listeria innocua* and *Bacillus cereus* was assessed by a well diffusion assay (Table 4). Sixteen strains showed antagonistic effects against pathogens: 5 of them inhibited one pathogen, 7 inhibited two and 5 inhibited the growth of the three pathogens tested. On the other hand, 9 strains showed no inhibitory effects at all. Fourteen strains were able to inhibit growth of *B. cereus*, whereas 12 of them also inhibited *E. coli* and only 7 strains showed anti-Listeria activity (Table 4). Regarding antimicrobials nature, 5 strains: *E. faecium* CRL 2150, CRL 2173, CRL 2176; *E. durans* CRL 2165 and *W. cibaria* CRL

Table 4
Technological and antimicrobial properties of LAB isolated from Argentinean kidney beans sourdoughs.

Strain	Amylolytic activity	Tannase activity	Gallate decarboxylase activity	Pathogens inhibition by cells free supernatants			BLIS production
				E. coli	*L. innocua*	*B. cereus*	
CRL 2147	−	−	+	+	−	+	−
CRL 2165	−	−	+	+	+	+	+
CRL 2148	−	−	−	+	+	+	+
CRL 2149	−	−	−	+	+	+	−
CRL 2150	−	−	+	+	+	+	+
CRL 2166	−	−	+	+	+	+	−
CRL 2180	−	−	+	+	−	+	−
CRL 2167	−	−	+	−	−	+	−
CRL 2170	−	−	−	−	−	−	−
CRL 2168	−	−	−	−	−	−	−
CRL 2169	−	−	−	−	−	−	−
CRL 2172	−	−	−	−	−	+	−
CRL 2171	−	−	−	+	+	−	−
CRL 2173	−	−	+	−	+	+	+
CRL 2174	−	−	−	−	−	+	−
CRL 2175	−	−	+	+	−	+	−
CRL 2176	−	−	−	+	−	+	+
CRL 2181	−	−	−	+	−	−	−
CRL 2182	−	−	+	+	−	+	−
CRL 2183	−	−	−	−	−	−	−
CRL 2184	−	−	−	−	−	−	−
CRL 2185	−	−	−	−	−	−	−
CRL 2177	−	−	−	−	−	−	−
CRL 2178	−	−	−	−	−	−	−
CRL 2179	−	−	−	−	−	−	−

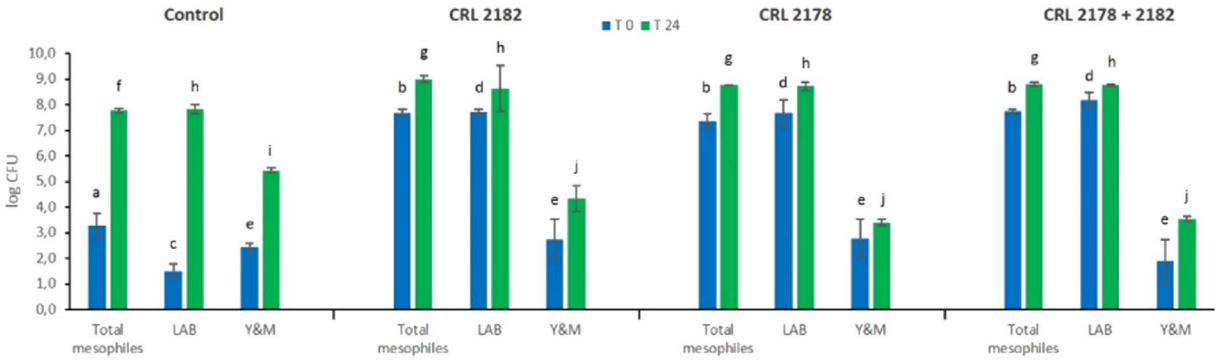

Fig. 4. Microbiological analyses of Alubia doughs inoculated with selected LAB strains and fermented for 24 h at 37 °C. Total mesophilic aerobic bacteria were estimated on Plate Count Agar (PCA), lactic acid bacteria on MRS agar; yeasts and molds on Yeasts and Molds selective Agar. Comparison between microbial groups was made at T0 and T24. Bars with different superscript letters (a, b, c, d, e, f, g, h, i and j) differ significantly ($P < 0.05$).

2148 produced inhibitory proteinaceous compounds (BLIS as they were not characterized for amino acid and nucleotide sequences), whereas other antagonistic substances were pH dependent and should be lactic and other organic acids (Table 4). *Enterococcus* species are well known bacteriocin producers (Khan, Flint, & Yu, 2010), however, BLIS production by *Weissella cibaria* isolated from sourdough is reported for the first time. Antimicrobial activity was a strain dependent feature: *E. faecium* CRL 2165 and *W. cibaria* CRL 2148 exerted antagonistic activity against the 3 pathogens tested whereas *E. faecium* CRL 2179 and *W. cibaria* CRL 2184 inhibited no pathogen. Other studies have reported the production of antibacterial and antifungal components by LAB from sourdoughs that can improve the safety and shelf life of end products and may represent a natural alternative to chemical preservatives in fermentation technology, meeting the consumers wishes for minimally processed foods (Corsetti & Settanni, 2007; Corsetti, Settanni, & Van Sinderen, 2004; Ruiz Rodríguez et al., 2016).

3.6. Fermentation of kidney beans flour with selected strains

Technological and functional properties of selected strains were assessed by their inoculation as single and mixed starters in kidney bean flour and fermentation during 24 h. Initial bacterial counts in spontaneous fermented dough, taken as control, were 3.28 ± 0.48, 1.50 ± 0.28 and 2.42 ± 0.17 log cfu/g for total mesophiles, LAB and yeast-molds respectively, whereas doughs inoculated with *W. paramesenteroides* CRL 2182, *E. durans* CRL 2178 and both strains combined contained around 7–8 log cfu/g of total mesophiles and LAB (Fig. 4). After fermentation, control dough reached 7.77 ± 0.10 log cfu/g for total mesophiles, 7.82 ± 0.19 log cfu/g for lactic acid microbiota and 5.43 ± 0.09 log cfu/g for yeast and molds. LAB inoculated sourdoughs reached 1 log higher counts for total mesophiles (8.77–8.99 log cfu/g) and LAB (8.63–8.77 log cfu/g) and 2 less log cycles for fungal microbiota (3.39–4.31 log UFC/g) with no differences ($P > 0.05$) among them.

3.7. Technological-functional properties of LAB sourdoughs

Before fermentation a mean pH of 6.05 and total titratable acidity of around 4.7 mL 0.1 NaOH/10 g was found for all doughs which is agreement with other legumes doughs (Curiel et al., 2015). Spontaneous fermentation for 24 h decreased pH to 5.67 ± 0.09 and increased TTA up to 18.40 ± 0.28. LAB inoculated sourdoughs showed ΔpH and ΔTTA values significantly higher than control ($P > 0.05$) (Table 5). *W. paramesenteroides* CRL 2182 showed the best acidification potential with final pH of sourdough of 4.55 ± 0.03 and 20.55 ± 0.35 mL 0.1 M NaOH/10 g of TTA.

Regarding proteolysis, it is known that released amino acids are converted into volatile compounds responsible for flavor of sourdough breads (Corsetti & Settanni, 2007). Then proteolytic microorganisms may improve the nutritional and organoleptics features of baked goods (Rizzello et al., 2014). Spontaneous fermentation of kidney bean dough during 24 h decreased FAA from 6.35 ± 0.60 to 3.25 ± 0.25 mmol Leu/g whereas sourdough inoculated with *E. durans* CRL 2178 increased FAA content to 7.15 ± 0.35 mmol Leu/g (Table 5).

Trypsin inhibitors are common ANF found in legumes that decrease proteins digestibility and affect the nutritional status of consumer. In the present study, we assessed the ability of selected starters to decrease concentration of this undesirable compound by fermentation. Although spontaneous fermentation decreased TIA (from 3.45 ± 0.60 to 2.21 ± 0.25 mg/g), LAB singly and combined were more efficient for removal of trypsin inhibitors from sourdoughs ($P < 0.05$) (Table 5). Sourdough containing both *W. paramesenteroides* CRL 2182 and *E. durans* CRL 2178 showed the greatest decrease of TIA from 3.50 ± 0.63 to 0.52 ± 0.35 mg/g. Other studies have shown that lactic fermentation decrease different ANF from legumes (Curiel et al., 2015; Granito et al., 2002).

Total phenols after fermentation were significantly ($P < 0.05$) higher in LAB sourdoughs compared to control, with the highest concentration found in dough fermented by *W. paramesenteroides* CRL 2182 (429 ± 58.00 mg GAE/g) (Table 5). Tannins are polyphenolic compounds that form complexes with dietary proteins decreasing their availability so that their removal would be desirable. A great decrease in tannin content was observed in LAB started sourdoughs with complete elimination after fermentation with CRL 2182 strain. Decrease of tannins and other ANF from faba bean flours was reported for *L. plantarum* (Coda et al., 2015) whereas this represents the first report for a *Weissella* species.

Antioxidant activity of Alubia sourdoughs at 24 h fermentation was determined as a relevant functional property with potential impact on health. Fermentation increased this activity in both control and LAB inoculated doughs. The higher DPPH scavenging activity was detected in sourdough fermented with both *W. paramesenteroides* CRL 2182 and *E. durans* CRL 2178 (68 ± 13.00%). Our results are in agreement with Curiel et al. (2015) who reported that sourdough fermentation of legume flours increased significantly the levels of antioxidant compounds by improving the bioavailability of phenolic compounds.

4. Conclusion

Legumes are an important source of nutrients and bioactive compounds, but in spite of their health benefits their consumption is below the recommended daily intake. Since health organizations recommend pulse consumption as a component of healthy diets, initiatives are being made to increase their cultivation, processing options and intake. One way for increasing their consumption could be the use of sourdough fermented legumes as health-enhancing ingredients in novel functional

Table 5
Technological and functional characteristics of control dough (spontaneous fermented dough) and sourdoughs (started with selected LAB) made with traditional Alubia kidney bean. Incubation was carried out at 37 °C for 24 h.

	ΔpH*	ΔTTA** (mL 0.1 NaOH/10 g)	Free amino acids (mM Leu/g)		Trypsin inhibitor activity (mg/g)		Total phenolics (mg GAE/g)		Tannins (mg GAE/g)		DPPH scavenging activity (%)	
			T 0	T 24	T 0	T 24	T 0	T 24	T 0	T 24	T 0	T 24
Control	0.39 ± 0.06^{a}	13.75 ± 0.21^{a}	6.35 ± 0.60^{a}	3.25 ± 0.25^{a}	3.45 ± 0.60^{a}	2.21 ± 0.25^{b}	325 ± 14.00^{a}	345 ± 7.00^{a}	4.33 ± 0.67^{a}	2.66 ± 0.48^{b}	45 ± 7.00^{a}	60 ± 14.00^{a}
CRL 2182	1.53 ± 0.06^{b}	16.05 ± 0.07^{b}	6.54 ± 0.40^{a}	5.21 ± 0.28^{ab}	3.50 ± 0.74^{a}	0.83 ± 0.07^{ab}	325 ± 14.00^{a}	429 ± 58.00^{a}	4.35 ± 0.64^{a}	0.00 ± 0.00^{a}	48 ± 6.00^{a}	63 ± 17.00^{a}
CRL 2178	1.28 ± 0.03^{bc}	14.7 ± 0.28^{bc}	6.50 ± 0.35^{a}	7.15 ± 0.35^{b}	3.48 ± 0.55^{a}	0.98 ± 0.21^{ab}	325 ± 14.00^{a}	396 ± 34.00^{a}	4.45 ± 0.64^{a}	0.28 ± 0.11^{a}	46 ± 8.50^{a}	55 ± 11.00^{a}
CRL 2178 + CRL 2182	1.41 ± 0.04^{c}	15.35 ± 0.21^{c}	6.30 ± 0.24^{a}	5.82 ± 0.21^{b}	3.50 ± 0.63^{a}	0.52 ± 0.35^{b}	325 ± 14.00^{a}	412 ± 59.00^{a}	4.35 ± 0.85^{a}	0.19 ± 0.27^{a}	48 ± 7.00^{a}	68 ± 13.00^{a}

$^{a–c}$: Means within each column with different letters are significantly different ($P \leq 0.05$).
* ΔpH: difference between pH values before and after fermentation.
** ΔTTA: difference between TTA values before and after fermentation.

foods, such as gluten free baked goods and snacks. Fermentation by LAB may improve sensory and functional characteristics of legumes and remove their anti-nutritional factors, but the right selection of microorganisms is a crucial step for this bioprocess. In the current study, we describe for the first time the LAB microbiota present in common beans cultivated and consumed in the north-western region of Argentina since they could be considered reservoirs of competitive LAB for functional purposes. Sourdough propagation allowed the isolation of promising microorganisms with appropriate technological, functional and safety properties that could be applied to the design of autochthonous starter cultures for the production of fermented legumes with added value. Two strains were rationally selected based on their properties: *Enterococcus durans* CRL 2178 (growth ability, acidification and proteolytic activities in beans extract) and *Weissella paramesenteroides* CRL 2182 (gallate decarboxylase and antimicrobial potential) as candidates for functional starter culture for legume flours fermentation. Sourdoughs obtained by simultaneous fermentation with both strains, combined technological and functional properties of each single strain. Further studies on the most appropriate combination ratio of strains and their application to manufacture of novel legume derived products are at present ongoing.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.09.042.

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