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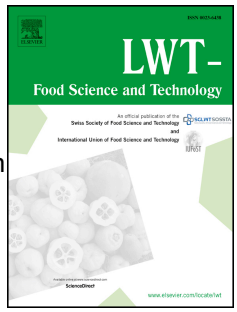

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IDENTIFICATION AND BIOTECHNOLOGICAL CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM CHICKPEA SOURDOUGH IN NORTHWESTERN ARGENTINA

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Running title: LAB from Argentinean chickpea sourdoughs

ABSTRACT

Chickpea, a relevant legume worldwide, can be nutritional and functionally improved by fermentation with lactic acid bacteria (LAB). In order to select suitable

autochthonous starter cultures, we isolated and identified LAB from kabuli chickpeas cultivated and consumed in northwestern Argentina, and screened their relevant techno-functional properties. Chickpeas were milled and spontaneously fermented with daily back-slopping at 37°C for 6 days and evolution of microbial populations were followed by plate counting. Phenotypic and genotypic methods including $(GTG)_5$-based PCR fingerprinting and 16S rDNA sequencing were used to differentiate and identify the isolates to species level. A marked increase of LAB counts was observed throughout fermentation raising from 0.88±0.35 log CFU/g of unfermented flours to 9.61±0.21 log CFU/g after 5 back-slopping steps with a concomitant pH decline from 6.09±0.05 to 4.40±0.03. Eighteen strains belonging to four LAB genera and six species: *Enterococcus durans, E. mundtii, Lactococcus garvieae, Pediococcus pentosaceus, Weissella cibaria* and *W. paramesenteroides* were identified in chickpea sourdoughs. Based on their abilities, *Weissella cibaria* CRL 2205 (acidification capacity), *W. paramesenteroides* CRL 2191 (proteolytic activity), *Pediococcus pentosaceus* CRL 2145 (gallate decarboxylase and peptidase activities), *Lactococcus garviae* CRL 2199 (α-galactosidase activity) and *E. durans* CRL 2193 (antimicrobial activity), were selected to design novel fermented chickpea products.

Keywords: Chickpea; legumes; sourdough; lactic acid bacteria; functional food.

1.- Introduction

Chickpea (*Cicer arietinum* L.) is one of the most relevant pulses for human consumption worldwide. According to the Food and Agriculture Organization (FAO), chickpea is a crop of importance in at least 50 countries (89.7% grown area in Asia,

4.3% in Africa, 2.6% in Oceania, 2.9% in the Americas and 0.4% in Europe) with a global production of 14.2 million tons per year. In Argentina, chickpea represents an interesting cool season crop option for irrigated areas and humid regions, such as northwestern foothills (Vizgarra, Espeche, & Ploper, 2013) in which, 60 thousand tons were produced last year and exported to the European Union, Turkey and Brazil (Source: Argentine Chamber of Legumes, http://www.clera.com.ar).

From a nutritional point of view, chickpea (like other legumes of the *Fabaceae* family) is unique, as it contains a higher proportion of protein (17%–30% by dry weight) than other plant foods. Besides, they provide consumers other nutrients and bioactive phytochemicals considered valuable for human health, such as complex carbohydrates, unsaturated fatty acids, dietary fiber, B-group vitamins, minerals and phenolic compounds with antioxidative properties. Their daily consumption has been associated with many health benefits such as the prevention of cardiovascular disease, diabetes, osteoporosis, gastrointestinal disorders, various cancers, hypercholesterolemia and obesity among others (Roy, Boye, & Simpson, 2010).

In addition, the functional properties of legume proteins (water binding capacity, fat absorption, foaming and gelation) and their gluten-free nature have increased the interest of using legume flours for the development of novel foods aided for celiac disease patients. Functional legume proteins from chickpea flour, have been used in the formulation of a wide variety of products such as pasta, bakery products and ready-to-eat snacks (Boye, Zare, & Pletch, 2010; Laleg, Cassan, Barron, Prabhasankar, & Micard, 2016; Miñarro, Albanell, Aguilar, Guamis, & Capellaset, 2012; Yagvci & Evci, 2017). However, chickpea, like the majority of legume plants have the capacity to synthesize antinutritional factors (ANF) such as amylase and protease inhibitors, phytic acid, saponins, tannins, lectins and α-galactosides, which reduce protein digestibility,

nutrient absorption and cause intestinal discomfort. Different food processing strategies such as soaking, dehulling, germination and long-time cooking are commonly applied to inactivate ANF, however some residual activity could remain and bioactivity of other phytochemicals could be affected (Patterson, Curran, & Der, 2017). Fermentation has proven to be an effective option for processing legumes that improve their nutritional and nutraceutical properties by the removal of ANF and the release of bioactive compounds (Coda et al., 2015; Curiel, et al., 2015; Chandra-Hioe, Wong, & Arcot; 2016; Gan, Shah, Wang, Lui, & Corke, 2016; Rizzello, Calasso, Campanella, De Angelis, & Gobbetti, 2014; Sáez, Hébert, Saavedra, & Zárate, 2017).

Fermentation can be spontaneously produced by endogenous microbiota of legumes (Gan et al., 2016; Rizzelo et al., 2014) or controlled by inoculation of starter cultures (Coda et al., 2015; Curiel et al., 2015; Gan et al., 2016; Chandra-Hioe et al., 2016; Sáez et al., 2017). Few studies have assessed the natural microbiota of unfermented and spontaneous fermented chickpea seeds and flours worldwide. Low numbers of mesophilic aerobic bacteria (1.6-4.3 log CFU/g) enterobacteria (0.5-0.9 log CFU/g), presumptive LAB (1.0-2.5 log CFU/g), yeasts (1.1-2.7 log CFU/g) and moulds (2.0–3.5 log CFU/g) were reported for Italian chickpeas and other legume flours (Curiel et al., 2015). Spontaneous fermentation significantly increase microbial populations but microorganisms vary according to regions and techniques used. Submerged fermentation of chickpeas from Syria and Greece were dominated by *Bacillus* and *Clostridium* species, whereas LAB were present in low numbers (Hatzikamari, Yiangou, Tzanetakis, & Litopoulou-Tzanetaki, 2007; Kyyaly, Lawand, & Khatib, 2017). Sourdough fermentation of Italian chickpea flours significantly increased LAB cell densities up to 8.6 log CFU/g after 5 days of backslopping propagation (Rizzelo et al., 2014). Microorganisms involved in fermented chickpea based foods produced in

Asia and Europe include *Leuconostoc, Lactobacillus, Streptococcus, Pediococcus, Micrococcus, Corynebacterium, Bacillus, Closstridium* spp. and yeasts (Hatzikamari et al., 2007; Katsaboxakis and Mallidi, 1996; Kyyaly et al., 2017; Rizzelo et al., 2014).

It is well known that LAB can improve organoleptic properties and safety of fermented products and that authochtonous strains usually perform better than allochtonous ones (Coda, Di Cagno, Gobbetti, & Rizello, 2014). In this sense, native LAB microbiota of the food matrix represents a natural reservoir of autochthonous cultures best suited for the fermented product; with diverse genetic information that might confer them high resistance to the manufacturing conditions and antagonistic activities that allow their dominance of the environment.

To our knowledge, no studies have addressed and characterized the lactic microbiota of Argentinean chickpeas neither their potential applications. In the present study, we isolated and identified LAB of kabuli chickpeas of northwestern Argentina and screened relevant techno-functional properties in order to obtain autochthonous cultures to design novel fermented legume products.

2. Materials and Methods

2.1. Sourdough preparation and acidity determinations

Five chickpea samples (*Cicer arietinum* var. kabuli*)* provided by local markets were individually milled to flours (Thermomix, Vorwerk, Madrid, Spain), mixed with tap water to obtain a dough yield [(dough mass/flour mass) × 100] of 200 and incubated for 24 h at 37°C. Five back slopping renewals were daily performed by inoculating at 10% (w/w) a fresh water-flour mixture with ripe sourdough from the day before. Ten grams samples from unfermented doughs (D0), and 1, 3 and 5 back-slopping steps

(BS1, BS3 and BS5) were taken for acidity and microbiological analysis (Sáez et al., 2017).

2.2. Microbiological analysis and lactic acid bacteria isolation

Each sourdough sample was homogenized during 3 min in Stomacher® 400 (Seward, Whorting, UK) with 90 mL of sterile 0.85% NaCl. Ten-fold serial dilutions were spread on selective agar media for isolation and count of different microorganisms: MRS (de Man, Rogosa & Sharpe; Britania, Argentina) supplemented with 0.1% ciclohexymide (Sigma, St. Louis), incubated for 48 h at 37°C under microaerophilic conditions, Rogosa agar (Oxoid, UK), 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 to 300 CFU were calculated, and at least 10 colonies grown in MRS and Rogosa agar with different morphologies were phenotypically characterized according to the Bergey Manual of Systematic Bacteriology, 8th edition.

2.3. Genotypic identification of the isolates

Chromosomal DNA was extracted from stationary phase cultures grown in MRS, and fingerprints of isolates were obtained by rep-PCR (Repetitive element (or extragenic) palindromic-Polymerase Chain Reaction) using the primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) and PCR reaction conditions as described in our previous report (Sáez et al., 2017). Resulting amplicons were separated by electrophoresis on 1.5% (w/v) agarose gels and visualized by UV transillumination after staining with GelRedTM Nucleic Acid Gel Stain (Biotium, Hayward, CA). Rep-PCR fingerprints

149 were analyzed using the software Bionumerics 7.50 (Applied Maths, Belgium) and
150 dendrogram was constructed applying the UPGMA algorithm.

151 Genotypic identification of isolates with different rep-PCR profiles was carried
152 out by partial 16S rDNA sequencing. Variable region V1 of the gene coding for 16S
153 ribosomal RNA was amplified with PLB16 (5'AGA GTT TGA TCC TGG CTC AG 3')
154 and MLB16 (5'GGC CAC TGC TGG GTA GTT AG 3') primers (Hebert, Raya,
155 Tailliez, & Savoy de Giori, 2000), purified and sequenced with an ABI 3130 DNA
156 sequencer (Applied Biosystems, Foster, CA, USA). Identification queries were fulfilled
157 by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) and in the
158 Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) and 16S rDNA sequences
159 were submitted to the European Nucleotide Archive database (LT965053 to
160 LT965070).

161

162 ***2.4. Techno-functional evaluation of the isolates: acidification capacity, proteolytic***
163 ***and amylolytic activities***

164 Technological properties of the isolates were assessed in a sterile flour extract
165 (SFE) used as liquid broth. Chickpea flour with the following proximate composition
166 (%): moisture, 7.87; ash, 3.96; protein 26.41; fat, 5.08; fiber, 2.05; carbohydrates, 3.50
167 (7.32 g/L sucrose, 11.23 g/L raffinose type oligosaccharides and 15.58 g/L stachyose);
168 was suspended at 40% (w/v) in distilled H_2O, stirred at 4°C during 2 hours and then
169 sterilized at 121°C for 20 min. Flour from the suspension was removed by
170 centrifugation (8000 rpm, 10 min at 4°C) and the supernatant was used as culture
171 medium (Sáez et al., 2107). LAB isolates developed in MRS were washed and
172 standardized in sterile 0.85% NaCl to OD_{560}: 0.8 and then inoculated in SFE (24 h at

37°C), taking samples at intervals for determinations of bacterial biomass, pH and free aminoacids (FAA) released by proteolysis.

Proteolytic activity of LAB was determined by the spectrophotometric assay of Church, Swaisgood, Porter, & Catignani, (1983). Samples were deproteinized with 0.75 mol/L trichloroacetic acid (1:2) and then supernatants were incubated with o-phthaldialdehyde (o-PA) solution (50 mL sodium tetraborate 100 mmol/L, 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 minutes at room temperature before reading their OD_{340}. The results were expressed as mmoles/L of FAA referring to a standard curve of L-leucine.

Amylolytic activity was determined by the ability of isolates to hydrolyze starch in agar. Active LAB were streaked on MRS agar plates containing 1% starch instead of glucose incubated at 37ºC for 48 h and then flooded with 4% (w/v) iodine solution. Amylase production was evidenced by the appearance of a clear zone around the colonies.

2.5. α-Galactosidase, proteinase and peptidase activities

Specific enzymes involved in degradation of legume's oligosaccharides and proteins were assayed in cell free extracts (CFE) by colorimetric methods. LAB cultures in 5 mL of SFE were centrifuged (10,000 g, 10 min, 4°C), washed twice with 100 mmol/L sodium phosphate buffer (pH 6.0) and resuspended in 0.5 mL of the same buffer (final $OD_{560} \cong 10$). Cells were disrupted with 500 mg glass beads (0.10–0.11 mm, Sigma) in a mini bead beater-8 (Biospec Products) for 10 cycles at maximum speed of 1 min each (with 1 min pauses on ice in between). Glass beads, cell debris and unbroken cells were removed by centrifugation (10,000 g, 5 min, 4°C) and the supernatant fluid

was used as CFE. Protein concentration was determined with a Bio-Rad protein assay kit based on Bradford procedure (1976).

α-Galactosidase activity was determined according to LeBlanc, Garro, & Savoy (2004) by monitoring at 410 nm the release of p-nitrophenol (pNP) from10 mmol/L of p-nitrophenyl-α-D-galactopyranoside (pNPG) used as substrate. 1 U α-gal was defined as the amount of enzyme that releases 1.0 μmol pNP/min and specific activity was expressed as U/mg of protein.

The presence of extracellular proteases was detected as described by Vermelho, Meirelles, Lopes, Petinate, Chaia, & Branquinha (1996). Active cultures were streaked on the surface of agar plates containing 1% (w/v) gelatin as substrate and incubated at 37°C for 48 h. Grown plates were flooded with 0.25% Coomassie blue in methanol-acetic acid-water 5:1:4 (v/v/v) and destained with methanol-acetic acid-water. A clear zone around the colonies was indicative of extracellular protease activity.

Aminopeptidase (AP) and endopeptidase (EP) activites were determined by the method of Rizzello et al. (2015). The colour intensity of the p-nitroaniline (p-NA) released from p-NA derivatives of L-anomers of leucine, lysine, and alanine, was measured at 410 nm. Endopeptidase (EP) activity was determined on N-succinyl L-phenyl-alanine-p-NA and N-glutaryl L-phenyl-alanine-p-NA as substrates. One U was defined as the amount of enzyme required to release 1 μmol of p-NA per minute under the assay conditions. Specific activity was expressed as U/mg of protein.

2.5. Tannase and gallate decarboxylase activity

The presence of enzymes involved in tannins metabolism were determined according to Osawa, Kuroiso, Goto, & Shimizu, (2000) with minor modifications. For tannase (E.C. 3.1.1.20), cultures grown on MRS agar were harvested with sterile swabs

and suspended at OD_{560}= 1 in NaH_2PO_4 (33 mmol/L) containing methylgallate (20 mmol/L); pH 5. Bacterial suspensions were incubated aerobically at 37°C for 24 h and then alkalinized with 2 mol/L $NaHCO_3$ solution (pH 8.6). Development of green to brown color of the medium was considered positive for tannase enzyme. For gallate decarboxylase activity (E.C. 4.1.1.59), active LAB cultures were inoculated at 1% (v/v) in MRS broth supplemented with 10 mmol/L gallic acid and incubated in anaerobiosis at 37°C for 72 h. Cultures were alkalinized with 2 mol/L $NaHCO_3$ solution (pH 8.6) and incubated aerobically at 37°C for 1 h. Development of dark yellow to brown color of the medium was taken as positive for gallate decarboxylase enzyme.

2.6. Antibacterial activity

Inhibition of foodborne bacteria by LAB isolates was determined by an agar well diffusion assay against *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 Laitières et Génétique Appliquée, INRA, France) used as sensitive target organisms (Sáez et al., 2107). Overnight cultures of LAB in MRS at 37 °C were centrifuged (6000 rpm, 10 min at 4 °C) and filtered through 0.22-µm pore-size filters in order to obtain cell-free supernatants (CFS). Aliquots of CFS were adjusted to pH 6.5 with NaOH, added with catalase (300 IU mL L^{-1}) or proteinase K (1 mg L^{-1}, 3 h at 37°C) to elucidate the nature of antimicrobial compounds produced (acid, H_2O_2 or bacteriocin, respectively). 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 hours. The appearance of inhibition halos around each well was taken as positive result for antimicrobials production.

2.7. Statistical analysis

The results are expressed as the mean ± standard deviation of five sourdoughs propagated in duplicate, and three assays ± sd for characterization of LAB isolates. Significant differences ($P < 0.05$) were determined by Tukey's test after analysis of variance (one-way ANOVA) with Minitab Statistic Program, release 14 for Windows.

3. Results and Discussion

3.1. Microbiological, pH and acidity analysis

Chickpea is a type of pulse crop with a long history of human health benefits. In view of these properties, its cultivation, processing options and intake have increased in recent years. In this respect, sourdough fermented legumes may be an interesting option for obtaining health-enhancing ingredients for novel functional foods such as gluten-free baked goods and ready-to-eat products (Rizzelo et al., 2014; Curiel et al., 2015; Chandra-Hioe et al., 2016; Sáez et al., 2017; Xiao et al., 2016). Fermentation by LAB may improve organoleptic and functional properties of pulses and remove their ANF, however, the right selection of microorganisms is a crucial step for this bioprocess. In consequence, in the present study, unfermented and spontaneous fermented chickpea flours (sourdoughs) were microbiologically analyzed after five back-slopping steps, for selecting autochthonous LAB as potential starter cultures for legume derived products. Figure 1 shows changes in the viable counts of most relevant microbial populations, pH and titratable acidity of chickpea sourdoughs after progressive fermentation. A significant increase ($P<0.05$) of total mesophiles and LAB counts were observed from the first day of fermentation raising from initial values of 4.57±0.34 and 0.88±0.35 log CFU/g of unfermented flours (doughs) to 10.40±0.39 and 9.61±0.21 log CFU/g after 5

back-slopping steps, respectively. LAB levels of unfermented chickpea flours were similar to those present in different varieties of Argentinean beans flours (Sáez et al., 2017) and in agreement with those reported for other flours (amaranth, rice, corn, quinoa, and potato) used in gluten free bread making (Corsetti et al., 2007).

Sourdough fermentation is a traditional food technology that exerts positive effects on technological and nutritional properties and shelf life of bakery products (Ganzle & Ripari, 2016). It is well-known that microbiota of stable sourdoughs of cereals and pseudocerals worldwide is mainly represented by LAB and yeasts (Corsetti & Settanni, 2007; Ganzle & Ripari, 2016; Huys, Daniel, & De Vuyst, 2013), although the dominance of microbial types and species found in mature sourdoughs may be influenced by temperature and flour characteristics (Minervini, De Angelis, Di Cagno, & Gobbeti, 2014). LAB counts of our chickpea sourdoughs attained the highest values at 5th back-slopping step and stabilized after 6 days of propagation (data not shown). These results are in agreement with other studies that reported “mature sourdoughs” (stable LAB consortium and acidification parameters) within 3 to 10 refreshments (Coda et al., 2017; Rizzelo et al., 2014; Vrancken, Rimaux, Weckx, Leroy, & De Vuyst, 2011).

Final LAB cell densities attained in chickpea sourdoughs (9.61±0.21 log CFU/g) were higher than the obtained by Rizzello et al., (2014) who reported median values of 8.6, 9.0, and 8.7 log CFU/g for chickpea, lentil and bean sourdoughs, respectively, after 5 days of propagation. In the same manner, we previously reported that LAB populations of Pallar, Alubia, Red and Black beans sourdoughs, reached 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, respectively (Sáez et al., 2017). However, LAB populations of our chickpea sourdoughs were similar

296 in LAB counts to that of mixed wheat-legume sourdoughs prepared for the manufacture
297 of Italian breads (Rizzelo et al., 2014).

298 Fungal counts (yeasts and moulds) decreased progressively from 3.84±0.42 at
299 the beginning of fermentation to 2.38±0.38 log CFU/g after the successive back-
300 slopping steps ($P<0.05$), remaining at levels of around 3 log CFU/g of sourdoughs at the
301 6^{th} day of fermentation. It has been reported that mature wheat sourdoughs incubated at
302 37°C contain yeasts population that do not exceed 5 log CFU/g (Coda et al., 2017).

303 The progressive dominance of LAB paralleled the decrease in pH and the
304 increase of titratable acidities of sourdoughs: pHs decreased from an initial value of
305 around 6.09±0.05 of unfermented doughs to about 4.40±0.03 after 6 days of propagation
306 by back-slopping reinoculation ($P<0.05$), whereas acidities raised from 4.46±0.21 to
307 21.84±0.29 mL of 0.1 mol/L NaOH per 10 g of sourdough ($P<0.05$). Similar results
308 were observed for Argentinean bean sourdoughs and Italian legume sourdoughs
309 fermented either spontaneously or LAB inoculated (Curiel et al., 2015; Sáez et al.,
310 2017).

311

312 ***3.2. Strain typing by rep-PCR***

313 Forty six colonies of different appearance grown in MRS and Rogosa agar plates
314 were picked and subjected to biochemical tests. Based on main phenotypic features
315 described in Bergey's Manual (non-motile bacilli and cocci, Gram positive, catalase and
316 nitrate negative microorganisms), 37 isolates were presumptively identified as LAB. All
317 isolates were subjected to rep-PCR $(GTG)_5$ fingerprinting technique for genotypic
318 grouping. The dendrogram constructed by UPGMA algorithm according to the
319 fingerprint band patterns obtained from the rep-PCR amplicons is shown in Figure 2.
320 The isolates were grouped in 9 clusters at a similarity level of 60%. Clusters I and II

presented high homogeneity in band profiles and contained strains that were isolated from unfermented flours and the first backslopping step (BS1) of different samples, suggesting adaptation and proliferation under similar environmental conditions. On the contrary, clusters III to VII showed high variability in band patterns that correlated with the diverse origin of isolates and fermentation times and contained the highest diversity of species that were further identified (Table 1). The remaining clusters VIII and IX also displayed a high similarity between band patterns, in spite of isolates coming from different backslopping cycles. Identical band patterns were considered as one rep-PCR profile which allowed grouping all isolates as belonging to 18 distinct profiles that were further identified by 16S rDNA sequencing. Partial 16S rRNA gene sequences obtained were compared with database at NCBI and RDP revealing high similarity values to: *Lactococcus garvieae* (2 strains), *Weissella cibaria* (5), *W. paramesenteroides* (4), *Enterococcus durans* (2), *E. mundtii* (*2*) and *Pediococcus pentosaceus* (3). The strains were deposited at CERELA Culture Collection with a CRL number assigned (Table 1) whereas their partial sequences of 16S rDNA were submitted to the European Nucleotide Archive database (Accession numbers LT965053 to LT965070).

Dynamics of LAB population and species composition in the chickpea sourdough ecosystem at each backslopping step is summarized in Table 2. Chickpea flours (day 0) were poor in LAB and only contained *Enterococcus durans* species whereas the following backslopping steps allowed development of a more complex microbiota. After the first refreshment step (BS1), other cocci such as *E. mundtii* and *Pediococcus pentosaceus* were isolated besides *Weissella paramesenteroides*. At third backslopping cycle, microbiota was further enriched by *Lactococcus garviae* and *Weissella cibaria*. Finally, after 6 days of fermentation, sourdoughs were dominated by *E. mundtii* and *Weissella* species. These results are correlated with those reported

346 previously for fermented beans flours, which included at the last sourdough stage, LAB
347 microbiota composed by *E. casseliflavus, Weissella cibaria* and *W. paramesenteroides*
348 (Sáez et al., 2017). It is well known that microbiota of traditional cereals and
349 pseudocereals (wheat, maize, sorghum, rye, quinoa and amaranth) sourdoughs is
350 dominated by LAB mostly belonging to *Lactobacillus* species such as *Lactobacillus*
351 *sanfranciscensis, L. plantarum, L. brevis* and *L. paralimentarius* (Coda et al., 2014;
352 Corsetti et al., 2007; Corsetti & Settanni, 2007; Ganzle & Ripari, 2016; Huys et al.,
353 2013; Ruiz Rodriguez et al., 2016). However, microbial diversity and stability may vary
354 depending on ecological factors like chemical and microbial composition of flour,
355 metabolic activities and interactions between microorganisms, the environment of
356 propagation and some specific technological parameters (e.g., percentage of inoculum,
357 time and temperature of fermentation), among others (Minervini et al., 2014; Vrancken
358 et al., 2011). Then, a wide range of LAB could be isolated from sourdoughs worldwide
359 and other genera such as *Leuconostoc, Weissella, Pediococcus* and *Enterococcus* have
360 also been identified (Corsetti & Settanni, 2007; Ganzle & Ripari, 2016; Huys et al.,
361 2013). Chickpea sourdoughs analyzed in this study contained 4 LAB genera and 6
362 species, a similar diversity to the previously found in Argentinean sourdough beans, but
363 no *Lactobacillus* was isolated by difference with them (Sáez et al., 2017).
364
365 ***3.3. Biotechnological properties of chickpea LAB isolates***
366 The application of LAB strains as starter culture for food fermentations requires
367 the analysis of specific properties relevant for the quality of the final product.
368 Acidifying, proteolytic and amylolytic activities of microorganisms could be desired
369 features since they contribute to preservation, safety, organoleptic and nutritional
370 characteristics of the product. In addition, the presence of enzyme activities involved in

ANF removal and/or the generation of bioactive metabolites could be taken as additional functional properties.

3.3.1. Growth, acidification and carbohydrates degradation

All strains were able to develop in chickpea SFE since bacterial biomass absorbances (OD_{560}) increased progressively whereas pHs decreased accordingly, reaching final pH values that ranged from 4.23±0.04 for *Weissella cibaria* CRL 2205 to 5.13±0.04 for *Pediococcus pentosaceus* CRL 2143 after 24 h of incubation (Table 3). None of the strains assayed showed ability to hydrolyze starch, and only two of them (*Enterococcus durans* CRL 2194 and *Lactococcus garvieae* CRL 2199) showed α-galactosidase activity. Then, most of the strains developed probably at expense of readily usable sugars like sucrose and monosaccharides present in chickpea SFE. α-Galactosidase that hydrolyzes α(1→6) linked sugars such as stachyose, raffinose and melibiose, has been described in LAB, bifidobacteria and fungi; and certain strains bearing this enzyme have been proposed for the removal of undesirable NDO and the relief of gastrointestinal discomfort associated to pulses consumption (Mansour & Khalil, 1998; LeBlanc et al., 2004).

3.3.2. Proteolytic activities

Proteolysis is relevant during pulses processing for improving the nutritional value, digestibility and bioactivity of their proteins and peptides. Primary proteolysis is dependent on substrate endogenous enzymes whereas LAB may release, during fermentation, small peptides and FAA by their strain-specific proteolytic systems. Since chickpeas have a high protein content, LAB proteolytic activities become relevant not only for their own development but also for the release of bioactive peptides and

essential AA that contribute to humans well-being and the flavor of fermented products. Proteolytic activities of the strains were determined indirectly by the quantification of FAA released in the chickpea-based growth medium, and directly by assessing protease and peptidase activities of LAB isolates. LAB strains displayed a wide range of proteolytic activities: seven isolates showed low abilities to release FAA (less than 1 mmol Leu/L) whereas the other 11 strains released 1 to 3 mmol Leu/L. *Weissella paramesenteroides* CRL 2191 showed the highest ability to release FAA (1.87 mmol Leu/L) (Table 4). Twelve strains displayed extracellular protease activity and three of them (*E. mundtii* CRL 2192, CRL 2196 and *L. garviae* CRL 2199) exhibited EP activity on Glu-Phe-pNA, whereas very low activity was recorded on Succ-Phe-pNA for all of the CFE assayed. Only four strains (*P. pentosaceus* CRL 2143, CRL 2144, CRL 2145 and *E. durans* CRL 2193) showed significant ($P<0.05$) AP activities (2.30±0.05 to 8.15±0.35 U/mg), being Lys-pNA the substrate hydrolysed at the highest rate (Table 4). *Pediococcus pentosaceus* CRL 2145 showed high activity on the three substrates tested with values of 6.02±0.21 U/mg for Leu-pNA, 6.64±0.13 for Lys-pNA and 4.56±0.10 U/mg for Ala-pNA. The four strains that express high AP showed greater activity when leucine or lysine was present at the N-terminal position with respect to alanine, which could be relevant for the release of these essential AA particularly high in pulses proteins (Boye et al., 2010). The proteolytic system of LAB isolated from legumes and its contribution to proteins degradation during fermentation has been scarcely studied. In a recent work, Verni et al. (2017) characterized the peptidase activities of LAB isolated from faba bean and revealed a large distribution of aminopeptidase PepN (especially in *Pediococcus* strains), which significantly increased FAA in fermented faba bean doughs. In agreement with this study, our chickpea LAB isolates displayed proteolytic activities that may contribute to chickpea bioprocessing.

421

422 *3.3.3. Tannase and gallate decarbolxylase activities*

423 Vegetable tannins present in many plants used as food and feed are considered
424 nutritionally undesirable since they inhibit digestive enzymes and affect the utilization
425 of vitamins and minerals. Tannase (E.C. 3.1.1.20) releases gallic acid from hydrolyzable
426 tannins whereas gallate decarboxylase (E.C. 4.1.1.59) decarboxylate gallic acid to
427 pyrogallol. Then, both enzymes could be relevant for removal of tannins and the release
428 of bioactive phenolic compounds (Muñoz et al., 2017). None of the strains assayed in
429 the present study showed tannase activity whereas 2 out of 18 strains: *Pediococcus*
430 *pentosaceus* CRL2145 and *Weissella cibaria* CRL2195 showed gallate decarboxylase
431 activity. Metabolism of tannins has been reported for *L. plantarum* (Muñoz et al., 2017)
432 and other LAB species such as *L. pentosus* and *L. paraplantarum* (Osawa et al., 2000)
433 whereas gallate decarboxilase activity was reported for *Enterococcus* (Nakajima, Otani,
434 & Niimura, 1992) and *Weissella* species isolated from different beans varieties (Sáez et
435 al., 2017).

436

437 ***3.4. Antibacterial activity of LAB from chickpea sourdoughs***

438 Vegetable fermentations and baked products are very sensitive to contamination
439 with spoilage and pathogenic microorganisms, which may represent a risk to public
440 health. Among them, *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus*
441 and *Bacillus cereus* are foodborne pathogens frequently reported (Law, Mutalib, Chan,
442 & Lee, 2014). LAB produce several antimicrobials such as organic acids, H_2O_2, and
443 bacteriocins, and have been proposed as a biopreservation strategy to counteract food
444 contamination during processing and a natural alternative to chemical additives (Reis,
445 Paula, Casarotti, & Penna, 2012). In the present study, antibacterial activity of the 18

LAB chickpea strains against *Escherichia coli*, *Listeria innocua* and *Bacillus cereus* was assessed as a safety property (Table 3). Fourteen strains showed antagonistic effects against the tested strains: 5 of them were able to inhibit one pathogen, 7 inhibited two and only 2 strains inhibited the growth of the three foodborne pathogens assayed. Eleven strains inhibited growth of *B. cereus*, 9 displayed inhibitory effects against *E. coli* and only 4 strains showed anti-*Listeria* activity (Table 3). Only two strains: *E. durans* CRL 2193 and *Weissella paramesenteroides* CRL 2198 produced bacteriocin-like inhibitory substances whereas other antagonisms were pH dependent and probably due to organic acids. Bacteriocin production is widespread among *Enterococcus* species (Khan, Flint, & Yu, 2010) and we have recently reported BLIS synthesis by *Weissella cibaria* CRL 2148 isolated from kidney beans sourdough (Sáez et al., 2017). Other studies have also reported the production of antibacterial and antifungal components by LAB from sourdoughs that can improve the safety and shelf life of final products (Corsetti & Settani, 2007).

4. Conclusion

To increase the consumption of chickpeas, new processing options and applications are needed. Fermentation with selected LAB may improve its nutritional, sensory and functional properties but the right selection of microorganisms is critical for successful outcomes. In the present study, we isolated and characterized for the first time the LAB microbiota present in chickpeas cultivated and consumed in the northwestern region of Argentina. Strains with promising properties: *Weissella cibaria* CRL 2205 (acidification capacity), *W. paramesenteroides* CRL 2191 (proteolytic activity), *Pediococcus pentosaceus* CRL 2145 (gallate decarboxylase and peptidase activities), *Lactococcus garviae* CRL 2199 (α-galactosidase and proteolytic activities) and *E. durans* CRL 2193 (antimicrobial potential), were selected to be applied as new functional starter

cultures for the production of fermented legumes with added value. The evaluation of the most appropriate combination of strains and their impact on the sensory and healthy properties of the final product is currently underway.

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Table 1: Genotypic identification by 16S rDNA sequence analysis of LAB isolated during chickpea sourdoughs fermentation

Isolate	CRL N°	Backslopping day	Dendogram cluster	Closest relative	% Identity	Submission N°
TG3	2191	1	VI	*Weissella paramesenteroides*	98%	LT965053
TG5	2143	1	II	*Pediococcus pentosaceus*	100%	LT965054
TG8	2144	1	VI	*Pediococcus pentosaceus*	100%	LT965055
TG14	2192	1	VIII	*Enterococcus mundtii*	96%	LT965056
TG22	2145	1	III	*Pediococcus pentosaceus*	99%	LT965057
TG36	2193	0	I	*Enterococcus durans*	100%	LT965058
TG53	2194	1	II	*Enterococcus durans*	99%	LT965059
TG83	2199	3	IX	*Lactococcus garvieae*	96%	LT965060
TG92	2200	3	VIII	*Lactococcus garvieae*	97%	LT965061
TG94	2195	3	V	*Weissella cibaria*	99%	LT965062
TG95	2196	5	VIII	*Enterococcus mundtii*	97%	LT965063
TG97	2197	5	IV	*Weissella cibaria*	92%	LT965064
TG123	2198	5	III	*Weissella paramesenteroides*	99%	LT965065
TG125	2201	3	IV	*Weissella paramesenteroides*	99%	LT965066
TG126	2202	3	VII	*Weissella cibaria*	99%	LT965067
TG127	2203	5	III	*Weissella cibaria*	99%	LT965068
TG138	2204	5	VII	*Weissella paramesenteroides*	99%	LT965069
TG139	2205	5	VII	*Weissella cibaria*	99%	LT965070

Table 2: LAB species identified at different back-slopping steps of chickpea sourdough fermentation

Back slopping cycle	*Enterococcus durans*	*Enterococcus mundtii*	*Lactococcus garvieae*	*Pediococcus pentosaceus*	*Weissella cibaria*	*Weissella paramesenteroides*
BS 0	■					
BS 1	■	■		■		■
BS 3			■		■	■
BS 5		■			■	■

Table 3: Some properties of LAB isolated from Argentinean chickpea sourdough

Strain	OD_{560}*	pH**	Amylolytic activity***	α-galactosidase activity□	Tannase activity†	Gallate decarboxylase activity‡	Bacterial inhibition by cells free supernatants§			BLIS production
							E. coli	*L. innocua*	*B. cereus*	
CRL 2191	2.63±0.11^{abc}	**4.48±0.04^{ab}**	-	Nd	-	-	+	-	+	-
CRL 2143	3.29±0.33^{cd}	5.13±0.04^{f}	-	Nd	-	-	+	-	+	-
CRL 2144	3.25±0.35^{cd}	5.09±0.08^{ef}	-	Nd	-	-	-	-	-	-
CRL 2192	2.27±0.06^{ab}	4.79±0.06^{cd}	-	Nd	-	-	+	+	+	-
CRL 2145	3.29±0.14^{cd}	5.11±0.04^{f}	-	Nd	-	+	+	-	-	-
CRL 2193	3.87±0.12^{d}	5.11±0.08^{f}	-	Nd	-	-	+	+	+	+
CRL 2194	2.10±0.14^{a}	4.95±0.07^{def}	-	13.96±0.60^{a}	-	-	+	-	+	-
CRL 2199	1.91±0.18^{a}	4.82±0.03^{cde}	-	**41.07±0.52^{b}**	-	-	-	-	+	-
CRL 2200	3.29±0.19^{cd}	4.82±0.05^{cde}	-	Nd	-	-	-	-	-	-
CRL 2195	3.07±0.10^{c}	5.00±0.17^{def}	-	Nd	-	+	-	-	-	-
CRL 2196	3.31±0.18^{cd}	4.80±0.08^{cd}	-	Nd	-	-	-	+	+	-
CRL 2197	3.14±0.20^{cd}	4.79±0.04^{cd}	-	Nd	-	-	-	-	+	-
CRL 2198	3.08±0.15^{c}	4.62±0.05^{bc}	-	Nd	-	-	+	+	-	+
CRL 2201	2.99±0.19^{bc}	4.76±0.01^{cd}	-	Nd	-	-	-	-	-	-
CRL 2202	3.17±0.03^{cd}	4.67±0.04^{bc}	-	Nd	-	-	-	-	+	-
CRL 2203	3.34±0.21^{cd}	4.61±0.02^{bc}	-	Nd	-	-	+	-	+	-
CRL 2204	2.13±0.18^{a}	4.88±0.06^{cdef}	-	Nd	-	-	+	-	+	-
CRL 2205	3.28±0.24^{cd}	4.23±0.04^{a}	-	Nd	-	-	+	-	-	-

Values are averages from three independent assays ± standard deviations. Means with different letter superscripts in the same column indicate significant differences (P<0.05). Selected strains are marked in bold.
*Absorbance of bacterial biomass after 24 h of growth on chickpea SFE.
**pH values of chickpea SFE after 24 h of LAB growth. Initial pH was 6.46.
***Amylolytic activity was expressed as (halo presence) or - (no halo around streak).
□ α-galactosidase activity was expressed as U mg^{-1}
† Tannase presence was expressed as + (green to brown color development) or – (light yellow).
‡ Gallate decarboxylase presence was expressed as + (dark yellow to brown color development) or – (light yellow).
§ Inhibitory activity was expressed as + (halo presence) or - (no halo around the well).
Nd: Not detected.

Table 4: Proteolytic activities of LAB isolated from Argentinean chickpea sourdough

Strain	FAA*	*Proteinase activity***	Aminopeptidase activity†			Endopeptidase activity	
			Ala-pNA	*Leu-pNA*	*Lys-pNA*	*Succ-Phe-pNA*	*Glu-Phe-pNA*
CRL 2191	**1.87±0.12^{d}**	+	0.20±0.04^{ad}	0.36±0.01^{a}	0.65±0.07^{e}	0.55±0.01^{a}	0.36±0.01^{e}
CRL 2143	1.69±0.28^{cd}	-	0.26±0.02^{af}	**3.19±0.22^{b}**	**5.27±0.21^{a}**	0.04±0.00^{e}	0.12±0.00^{h}
CRL 2144	0.81±0.16^{ab}	-	**2.30±0.05^{b}**	**3.64±0.07^{b}**	**4.19±0.28^{b}**	0.31±0.00^{c}	0.11±0.01^{h}
CRL 2192	0.94±0.08^{ab}	+	0.28±0.04^{af}	0.47±0.04^{c}	0.29±0.02^{f}	0.27±0.02^{c}	**7.23±0.13^{a}**
CRL 2145	0.42±0.03^{a}	-	**4.56±0.10^{c}**	**6.02±0.21^{d}**	**6.64±0.13^{c}**	0.23±0.01^{d}	0.20±0.01^{f}
CRL 2193	1.34±0.26^{bcd}	-	0.15±0.03^{d}	**5.71±0.11^{d}**	**8.15±0.35^{d}**	0.07±0.01^{f}	0.26±0.00^{g}
CRL 2194	0.90±0.11^{ab}	+	0.09±0.06^{d}	0.37±0.04^{a}	0.63±0.07^{e}	0.47±0.01^{b}	0.65±0.09^{d}
CRL 2199	1.69±0.21^{cd}	+	0.70±0.06^{e}	0.96±0.07^{e}	0.65±0.10^{e}	0.54±0.01^{a}	**3.48±0.32^{b}**
CRL 2200	0.84±0.06^{ab}	+	0.33±0.07^{f}	0.55±0.06^{c}	0.30±0.08^{fgi}	0.13±0.01^{g}	0.31±0.03^{g}
CRL 2195	1.21±0.18^{bc}	+	0.00±0.00^{g}	0.15±0.05^{f}	0.21±0.04^{g}	0.13±0.01^{g}	0.23±0.04^{fg}
CRL 2196	1.70±0.09^{cd}	+	0.18±0.04^{ad}	0.32±0.01^{a}	0.28±0.01^{f}	0.16±0.01^{h}	**6.19±0.25^{c}**
CRL 2197	0.90±0.07^{ab}	+	0.00±0.00^{g}	0.19±0.06^{fgh}	0.29±0.03^{f}	0.17±0.01^{h}	0,24±0.02^{fg}
CRL 2198	0.98±0.12^{ab}	+	0.20±0.02^{ad}	0.09±0.03^{g}	0.10±0.01^{h}	0.03±0.00^{e}	0,19±0.01^{f}
CRL 2201	1.13±0.09^{bc}	+	0.07±0.05^{d}	0.27±0.01^{h}	0.28±0.02^{f}	0.13±0.01^{g}	0,25±0.04^{fg}
CRL 2202	1.34±0.19^{bcd}	-	0.20±0.07^{adf}	0.48±0.02^{c}	0.43±0.04^{i}	0.23±0.01^{d}	0,28±0.02^{fg}
CRL 2203	1.26±0.08^{bcd}	-	0.09±0.03^{d}	0.20±0.01^{f}	0.30±0.01^{f}	0.22±0.01^{d}	0,16±0.01^{i}
CRL 2204	1.69±0.11^{cd}	+	0.07±0.02^{d}	0.13±0.01^{g}	0.11±0.01^{h}	0.04±0.00^{e}	0,07±0.01^{j}
CRL 2205	1.65±0.18^{cd}	+	0.07±0.04^{d}	0.22±0.02^{g}	0.25±0.02^{g}	0.15±0.01^{g}	0,15±0.01^{i}

Values are averages from three independent assays ± standard deviations. Means with different letter superscripts in the same column indicate significant differences ($P<0.05$). Selected strains are marked in bold.
*Indirect proteolytic activity determined by FAA quantification (millimols L-Leu/L) in chickpea SFE after 24 h of LAB growth.
**Extracellular proteinase activity was detected by hydrolysis of gelatin and expressed as + (halo presence) or - (no halo around streak).
† Exopeptidase (aminopeptidase) and Endopeptidase activities were expresed as U mg^{-1} released from each substrate.

FIGURE LEGENDS

Figure 1: Microbiological analyses [total mesophilic aerobic bacteria (●); lactic acid bacteria (■) and yeasts and molds (▼)], pH (△) and titratble acidity (○) of Argentinian chickpea flours before (doughs) and after 5 back-slopping steps (sourdoughs) incubated for 24 h at 37°C. The data are the means of five determinations ± standard deviations of two independent assays. Mean values of each curve not sharing the same letter are different at $P<0.05$.

Figure 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 rDNA sequencing.

Figure 1

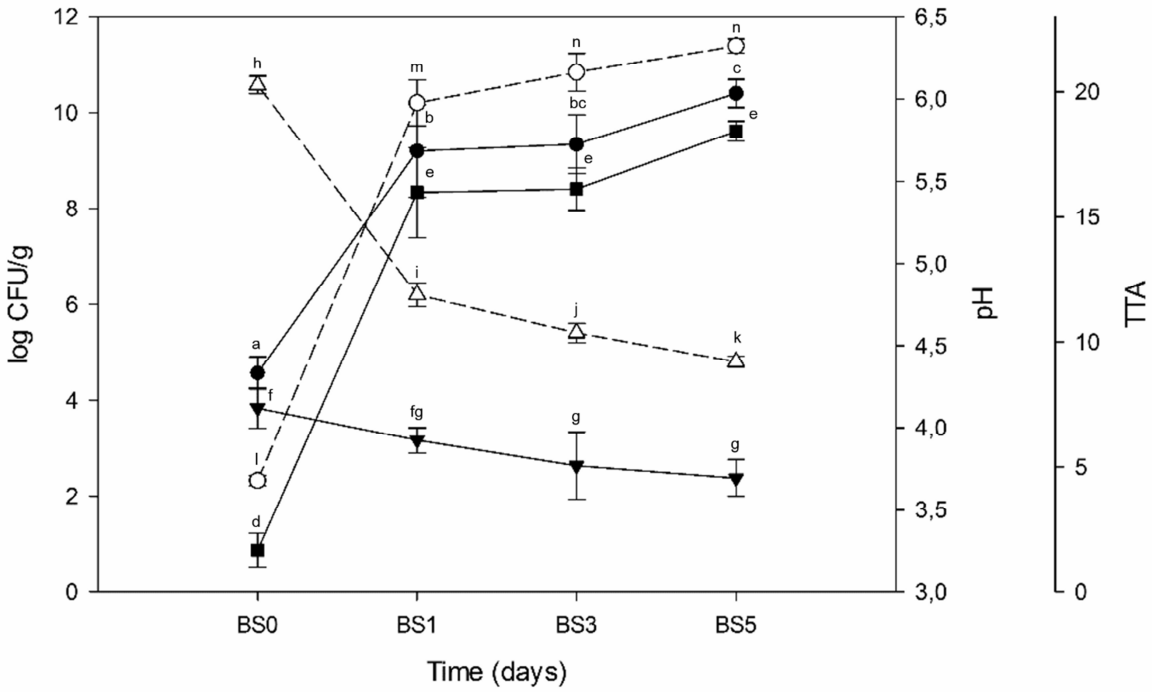
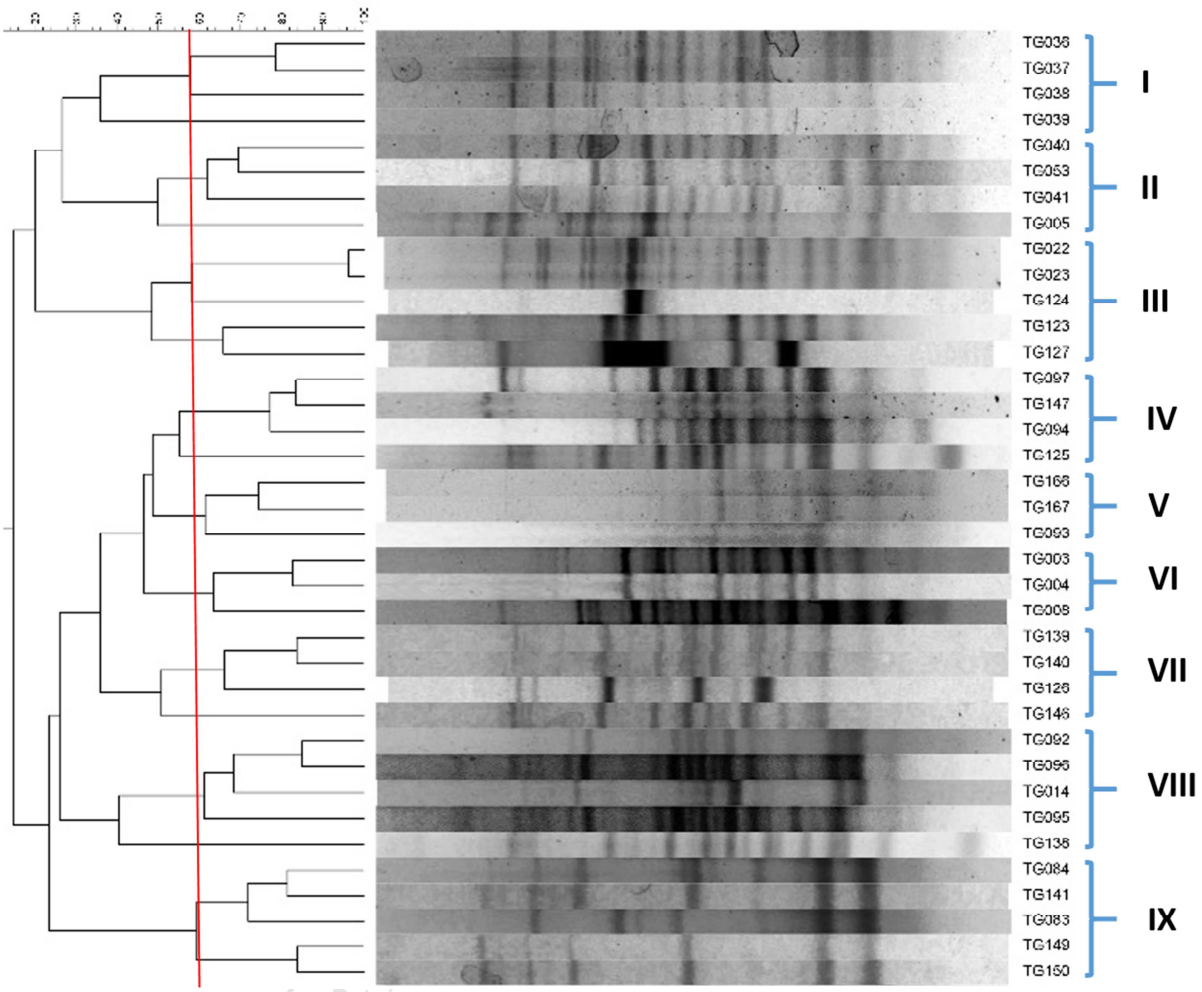


Figure 2



Highlights

- Argentinean kabuli chickpeas were microbiologically analyzed for LAB isolation.
- Six LAB species were identified by Rep-PCR fingerprinting and 16S rDNA sequencing.
- Some strains have potential to improve nutritional quality and safety of legumes.
- Five LAB strains were selected for developing novel fermented chickpea derived foods.