Lactic Acid Bacteria from Argentinean Fermented Foods: Isolation and Characterization for their Potential Use as Starters for Fermentation of Vegetables

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SUMMARY
Lactic acid bacteria (LAB) improve the organoleptic, nutritional and physicochemical properties of artisanal foods. In this study, we selected 31 fermented dairy and vegetable foods marketed in Tucumán city, Argentina, as sources of LAB for the production of pickles. Sixty-four isolates presumptively identified as *Lactobacillus* strains were screened for relevant technological properties for production of fermented foods. Most strains showed moderate to good acidification (>0.04 pH units/h) and proteolytic capabilities (free aminoacids >1 mmol/L), produced diacetyl and/or acetoin and were resistant to 4 % NaCl. Based on acid production and osmotolerance, we selected six LAB strains and identified them by 16S rDNA sequencing (97–100 % identity) as: *Lactobacillus rhamnosus* CRL2159 and CRL2164, *L. plantarum* CRL2161 and CRL2162, *Weissella viridescens* CRL2160 and *W. paramesenteroides* CRL2163. Relevant properties for pickle manufacturing were further assessed. At an initial pH=4.5 and 7 % NaCl, *L. plantarum* CRL2162 and *L. rhamnosus* CRL2164 performed the best with high growth and inhibitory activity against *Escherichia coli* and *Listeria innocua*. There was no obvious antagonism among the selected strains that would dismiss their use in mixed cultures. Properties of the selected LAB suggest their potential as starter cultures for obtaining standardized, fermented vegetable products of high quality. The development of these new industrial starters would increase the competitiveness of production and open the country’s frontiers in the canned vegetable market.

Key words: fermented vegetables, lactic acid bacteria, starter culture, pickles

INTRODUCTION
Fermentation is a biological process widely used for food production and preservation. In recent decades, the fermentation technology used in food manufacturing has moved from artisanal practices to industrialized biotechnological processes that allow for the production of high quality standardized products. This change is possible because of the usage of starter cultures, which are pure or mixed cultures of microorganisms inoculated into a food matrix in order to replace its endogenous microbiota and improve the fermentation by enhancing nutritional and functional properties, organoleptic characteristics and safety (1).

The main microorganisms involved in industrial fermentations are yeasts and bacteria whose selection depends on their final metabolites. Lactic acid bacteria (LAB) are the most desirable for food manufacturing due to their technological and probiotic properties, whereas other genera, such as *Propionibacterium* (2) and *Acetobacter* (3), are used for the production of propionic and acetic acids, respectively.

When selecting LAB for food fermentations, different technological properties that could be relevant for the final product must also be considered. This properties include the acidifying, proteolytic and lipolytic activities that contribute to the preservation, flavour and nutritional quality of the product. Additionally, the presence of enzymatic activities involved in the removal of antinutritional factors (ANF), and the protection of consumers from their adverse effects is a desired feature. Resistance to different stressful conditions, such as high salt concentrations, development at acidic pH and growth at high or low temperatures, are also considered as selection criteria for the development
of starters for the food industry. Vegetables are an important niche for the isolation and selection of LAB for starter and probiotic applications (4,5). The fermented plants most widely consumed around the world include olives, cucumbers and cabbage, and to a lesser extent carrots, beets and peppers. Eastern countries also produce fermented products that incorporate radishes, turnips, Brussels sprouts, and legumes (6).

In Argentina, canned vegetables and fermented artisanal foods such as pickles are well-accepted products consumed as an appetizer or a side dish. The manufacture of pickled vegetables involves spontaneous fermentation, leading to the dominance of lactic acid microbiota. The acid produced by these microorganisms eliminates both spoilage bacteria (mainly enterobacteria and sporulated bacteria) and their pectinolytic enzymes responsible for putrefaction. LAB that dominate primary fermentation include Enterococcus faecalis, Lactobacillus plantarum, Leuconostoc mesenteroides, Lactobacillus brevis and Lactobacillus sakei. However, the first two species do not resist high salt concentrations or acidification and rapidly decline, whereas lactobacilli complete the majority of vegetable fermentations (5). Although many fermented vegetable products are on the market in northwestern Argentina, their artisanal production without the addition of starter cultures makes them an important reservoir of autochthonous LAB with the potential for further applications. The development of industrial starters for fermentation of pickles would offer products with more appreciable organoleptic properties and a longer shelf-life, and would provide an economic alternative within the food chain. This would increase the competitiveness of production, leading to the opening of new frontiers in the canned vegetable market. Therefore, the objective of this work is to isolate and select LAB strains from different artisanally fermented products for the formulation of a starter culture applicable to pickle fermentation. For this purpose, we evaluated a series of technological properties relevant for this type of fermented food production and the compatibility of the selected strains.

MATERIALS AND METHODS

Isolation of lactic acid bacteria from artisanally fermented foods

Thirty-one artisanally fermented products (pickles made from carrots, peppers, onions and cabbages; olives and cheese made with unpasteurized milk) came from different local markets in San Miguel de Tucumán city, Argentina. Samples (10 g) were homogenized in 90 mL sterile 0.9 % (m/v) NaCl, 10-fold diluted and plated at 37 °C and 5 % CO2 for 72 h on MRS agar (composition in g/L: meat peptone 10, beef extract 10, yeast extract 5, glucose 20, sodium acetate 5, ammonium citrate 2, K2HPO4 2, Tween 80, MgSO4·7H2O 0.57, MnSO4·4H2O 0.12, FeSO4·0.034 and agar, 15 pH=5.4, with glacial acetic acid; Oxoid, Basingstoke, UK) selective for lactobacilli. Colonies selected by their different morphology and texture were transferred to LAPtg broth (composition in g/L: meat peptone 15, tryptone 10, yeast extract 10, glucose 10 and Tween 80 1, pH=6.5; Laboratorios Britania SA) and their phenotypic evaluation followed the methods of Kandler and Weiss (7). Gram-positive, catalase-negative and nitrate reductase-negative bacilli were selected for further characterization.

Technological properties of isolates

Grown cultures were standardized using a Spectroscopic 20 spectrophotometer (Bausch & Lomb, Rochester, NY, USA) to absorbance A560 nm=0.8 in phosphate-buffered saline (Laboratorios Cicarelli® Santa Fe, Argentina), inoculated at 2 % rate into 10 % (m/v) reconstituted skimmed milk (La SerenaSima, Buenos Aires, Argentina) and incubated at 37 °C for 24 h.

The acidification capacity of the microorganisms was determined by pH (Altronix TPX IPh meter; Brooklyn, NY, USA) and titratable acidity measurements at 12 and 24 h of incubation (8).

Proteolytic activity of isolates was determined using the spectrophotometric assay of Church et al. (9). Samples were deproteinized with 0.75 M trichloroacetic acid (1:2) (Laboratorios Cicarelli®) and then supernatants were incubated in an o-phthalaldehyde (o-PA) solution (50 mL of 100 mM sodium tetraborate; Merck, Darmstadt, Germany; 5 mL of 20 % (m/v) sodium dodecyl sulphate; Merck; 2 mL of 40 mg/mL o-PA dissolved in methanol, and 0.2 mL β-mercaptoethanol; Sigma-Aldrich, Merck, St. Louis, MO, USA) for 10 min at room temperature before reading the absorbance at A540 nm with a UV-visible spectrophotometer (Varian, Agilent Technologies, Santa Clara, CA, USA). The results were expressed in mmoles of free amino acids (FAA) per litre of milk by referring to a standard curve of l-leucine. Activities were classified as low, intermediate and high (0–1, 1–2 and >2 mmol/L, respectively).

Amylolytic activity was determined by the ability of bacteria to hydrolyse starch in agar. Active LAB were streaked on LAP agar plates containing 1 % starch instead of glucose, incubated at 37 °C for 48 h, and then flooded with a 4 % (m/v) iodine solution (Laboratorios Britania SA). The appearance of a clearing zone around the colonies confirmed amylase production and the diameter of these halos was measured with a calliper (10).

Medina et al. (11) assessed lipolytic activity using the tributyrin agar diffusion assay. Agar plates containing 20 mL medium composed of: 1.5 % (m/v) agar–agar, 0.25 % (by volume) triglycerides, 0.02 % (m/v) sodium azide and 100 mmol/L sodium phosphate buffer, pH=7.0 (Laboratorios Cicarelli®) were prepared and once solidified, wells of 1 cm in diameter were made on the agar. Aliquots of cell suspensions (100 µL) were inoculated into the wells and activity was observable as a detectable zone of hydrolysis (halos) after 48 h of incubation at 37 °C.
Diacetyl and/or acetoin production was determined qualitatively from coagulated milk samples. A 1-mL aliquot was mixed with 0.5 mL of 0.5% (m/V) α-naphthol and 0.2 mL KOH-creatine (Sigma-Aldrich, Merck) in 40% KOH (Laboratorios Cicarelli®) for 15 s and allowed to stand for 5 min. The appearance of a red ring at the top of the tube represented positive result (11).

Tolerance to acid and saline stresses was evaluated by assessing the growth of microorganisms in LAPtg broth acidified with lactic acid (Sintorgan SA, Buenos Aires, Argentina) to pH=5.5, 5.0, 4.5 or 4.0, or supplemented with 2, 4, 7 or 10% (m/V) NaCl (Laboratorios Cicarelli®), respectively. Growth was determined by the increase of bacterial biomass absorbance (A_{600nm}) measured at intervals during 24 h of incubation at 37 °C. The experimental data of the growth curves were adjusted using the reparameterized Gompertz model (12) expressed by the following function (13):

\[ A_t = A_b + A_m \exp\{-\exp[(\mu e/A)(t-t_0)+1]\} / I \]

where \( A_b \) is absorbance at time \( t \), \( t \) is time of growth in hours, \( A_m \) is absorbance at \( t=0 \), \( A \) is increase of molar absorption coefficient between \( A_b \) and \( A_m \), \( \mu \) is maximum growth rate (h^{-1}), \( t_0 \) is duration of time of lag phase in hours, and \( e \) is base of Napierian logarithm (2.718281828). Calculated parameters were considered relevant within the bacterial growth dynamic as they reflect the behaviour of strains under each one of the assayed conditions.

**Genotypic identification of strains**

The 16S ribosomal RNA gene was sequenced to identify the microorganisms. Total genomic DNA extraction followed the protocol described by Pospiech and Neumann (14). Amplification of variable region V1 of the 16S ribosomal RNA gene was performed by polymerase chain reaction (PCR) using primers PLB16 (5’ AGA GTT TGA TCC TGG CTC AG 3’) and MLB16 (5’ GCC TG C TGG CAC GTA GTT AG 3’) (15). The amplification programme was carried out in a thermocycler (MyCicler, BIORAD, Hercules, CA, USA), and consisted of a 5-minute initial denaturation at 94°C, followed by 30 cycles at 94°C (30 s), 52°C (30 s) and 72°C (45 s), and finally an elongation step of 10 min at 72°C. The amplicons obtained from the reaction were purified with PEG (20% polyethylene glycol 8000 in 2.5 M NaCl; Sigma-Aldrich, Merck) and sequenced using an ABI 3139 DNA sequencer (Applied Biosystems, Foster, CA, USA) (16). The identity of the sequences was determined by comparison with sequences deposited in online databases: BLAST GenBank and Ribosomal Database Project (17, 18). The sequences of the partial 16S rRNA of LAB were submitted to the European Nucleotide Archive database (accession numbers LT714201 to LT714206) (19).

**Tannase and gallate decarboxylase activities**

The presence of enzymes involved in antinutritional tannin metabolism was determined according to the method of Osawa et al. (20) with some modifications (21). For tannase (EC 3.1.1.20) activity, cultures grown on MRS agar were harvested with sterile cotton swabs and suspended up to absorbance value of A_{600nm}=1 in 33 mmol/L NaHPO_4 (Laboratorios Cicarelli®) containing 20 mmol/L methylgallate (Sigma-Aldrich, Merck), pH=5. Bacterial suspensions were incubated aerobically at 37 °C for 24 h and then alkalinized with a 2 M NaHCO_3 solution (pH=8.6; Laboratorios Cicarelli®). After exposure to air for 1 h at room temperature (25 °C), the development of a green to brown colour of the medium was considered a positive result for tannase enzyme. For gallate decarboxylase (EC 4.1.1.59) activity, overnight cultures were inoculated at 1% (by volume) in MRS broth supplemented with 10 mmol/L gallic acid (Sigma-Aldrich, Merck) and incubated anaerobically at 37 °C for 72 h. Cultures were then alkalinized with a 2 M NaHCO_3 solution (pH=8.6) and incubated aerobically at 37 °C for 1 h. Development of a dark yellow to brown colour was taken as a positive result for gallate decarboxylase enzyme.

**Inhibition of pathogens**

The ability of the isolates to produce antimicrobial substances was determined using an agar well diffusion assay (21). Two common food pathogens, *Escherichia coli C3* (from Institute of Microbiology “Luis Verna” of University of Tucumán, Argentina) and *Listeria innocua 7* (from Unité de Recherches Laitières et Génétique Appliquée, INRA, Jouy-en-Josas, France) were used as indicator strains. Overnight cultures of LAB grown at 37 °C were harvested by centrifugation (5000g, 10 min at 4 °C) in a tube centrifuge (Presvac, Buenos Aires, Argentina) and filtered through 0.22-μm pore size filters (Biopore SRL, Buenos Aires, Argentina) to obtain cell-free supernatants. To elucidate the nature of antimicrobial compounds, aliquots of cell-free supernatants were adjusted to pH=6.5 with 1 M NaOH (Laboratorios Cicarelli®), and treated with catalase (300 IU/mL) or proteinase K (1 mg/L, 3 h at 37 °C) (Sigma-Aldrich, Merck) in order to discriminate acid, H_2O_2 or bacteriocin inhibition. Treated and untreated cell-free extracts were inoculated (50 μL) in wells made in brain heart infusion (BHI) agar plates (Laboratorios Britannia SA) seeded with each pathogen and incubated at 37 °C for 24 h. The appearance of inhibition halos around each well was taken as a positive result for production of antimicrobials.

**Compatibility of strains**

**Agar diffusion assay**

Cell-free supernatants of overnight cultures of selected LAB were inoculated in wells made in LAPtg agar plates previously seeded with each of the other strains. The plates were incubated at 37 °C for 48 h, the presence of growth inhibitory halos was considered as an incompatibility between strains.

**Cross-streak assay**

Overnight cultures were washed (10 000xg, 10 min, 4 °C) with sterile saline solution (0.9% m/V NaCl) and seeded on LAPtg agar in a straight line using a sterile swab. Cultures of
the other strains were seeded perpendicular to the first line. After 72 h of incubation at 37 °C, the type of growth in the confluence zones (stimulation, inhibition, or absence of interaction between the strains) was visually determined (22).

**Statistical analysis**

The results are expressed as the average of three independent trials±standard deviation for the characterization of LAB isolates. Significant differences between mean values were determined by Tukey’s test after analysis of variance (one-way ANOVA) with the Minitab v. 17 for Windows (23). A p<0.05 was considered statistically significant. Technological properties of strains were analysed through principal component analysis using the XLSTAT software v. 19.4 (24). The Nonlinear Estimation module included in Statistica v. 13.3.0 (25) was applied to fit the growth curves and calculate growth parameters included in the Gompertz mathematical model (12).

**RESULTS AND DISCUSSION**

**Isolation and phenotypic characterization of lactic acid bacteria**

Although there is a great variety of fermented foods, most commercial LAB starter cultures are developed for the dairy industry and to a lesser extent for sausage production. Fermented vegetables are traditionally obtained through a process of spontaneous lactic acid fermentation, which is highly dependent on the epiphytic microbes present on the raw materials (4,26). At present, there are few industrial starters for pickle fermentation worldwide and no autochthonous LAB starter for this purpose in Argentina that could give competitive high quality products. Therefore, in the present study, we isolated and technologically characterized the endogenous lactobacilli present in 15 dairy (artisanal cheese), olives and 15 vegetable (pickles) fermented products that are marketed in the streets of San Miguel de Tucumán city located in Tucumán Province in the northwestern region of Argentina. We obtained a total of 78 isolates, and presumptively identified 64 according to phenotypic characteristics (Gram (+), catalase (−), nitrate reductase (−), bacilli) as belonging to the genus *Lactobacillus*, one of the most representative among LAB. In regards to the origin of the bacteria, similar proportions of LAB isolates were obtained from both environments (53 % from dairy and 47 % from pickles). To select LAB with optimal characteristics to be used as starter cultures, all of the isolates were technologically characterized concerning their relevant properties for use in fermented foods.

**Technological properties**

The technological properties of microorganisms from dairy and pickles are summarized in Table 1. The isolates of dairy origin displayed the best acidifying properties. A high proportion of isolates (64 %) were able to coagulate milk between 12 and 24 h and to decrease pH below 5, and they showed high acidification rates. Isolates from fermented products were divided into strains that coagulate milk between 12 and 24 h (50 %) and others that were unable to coagulate milk up to 36 h (50 %). Accordingly, more than 50 % of the isolates showed low acidification rates. However, the percentage of strains with high acidification rates (approx. 30 %) was similar in both environments (Table 1). A rapid and high production of acid is demanded for lactic cultures to be used as starters in plant fermentation technology. Different studies have assessed the acidification activity of LAB from vegetables. *L. plantarum* KJ03 and *L. fermentum* KJ23 isolated from fermented stink beans (sataw-dong) reached pH=3.86–3.97 and titratable acidity of 1.53–1.55 % after 24 h of growth (27), whereas *L. plantarum* 25 and *L. pentosus* 13 isolated from pepper and beetroot pickles may be preferable as starter cultures for pickle and olive fermentations in Turkey because they produce high acid levels and most of the acid production is on the first day of fermentation (28). In our study, isolates GS25 and GS43 of dairy origin and GS31 and GS109 from olives and pickles, respectively, reached the lowest pH after 24 h (3.97±0.02 to 4.03±0.02) and the highest acidification rates (Table 2).

**Table 1.** Technological properties of Argentinian lactic acid bacteria (LAB) isolates distributed according to their origin

| Property | wLAB/% | Origin | Pickle |
|----------|--------|--------|--------|
| Time (milk coagulation)/h | | | |
| <12 | 0 | 0 | |
| 12–24 | 64 | 50 | |
| No coagulation up to 36 | 36 | 50 | |
| Final pH | | | |
| >6 | 15.7 | 50 | |
| 5.1–6.0 | 21 | 12.5 | |
| <5.0 | 63 | 37.5 | |
| Acidification rate | | | |
| Low (0–0.04) | 26.3 | 57 | |
| Medium (0.04–0.08) | 42.1 | 14 | |
| High (>0.08) | 31.6 | 29 | |
| Proteolytic activity | | | |
| Low (0–1) | 23 | 50 | |
| Medium (1–2) | 18 | 50 | |
| High (>2) | 59 | 0 | |
| Diacetyl or acetoin production | | | |
| No production | 27 | 54 | |
| Low | 29 | 23 | |
| Medium | 32 | 23 | |
| High | 12 | 0 | |
| Osmotolerance | | | |
| w(NaCl)=2 % | | | |
| Low (<50 % Acontrol) | 6 | 17 | |
| High (>50 % Acontrol) | 94 | 83 | |
| w(NaCl)=4 % | | | |
| Low (<50 % Acontrol) | 68 | 90 | |
| High (>50 % Acontrol) | 32 | 10 | |

*aFinal pH measured at coagulation time, acidification rate expressed as ΔpH/h, ΔpH=PHfinal–PHinitial, proteolytic activity, expressed as mmol of free amino acids per litre of milk, growth expressed as percentage of Acontrol in media without NaCl*
Table 2. Lactic acid bacteria strains isolated from fermented artisanal products marketed in Tucumán city, Argentina, with the best technological properties

| Isolate | Source (location) | Initial pH | pH (t=12 h) | pH (t=24 h) | ΔpH | Acidification rate (ΔpH/h) | Coagulation\(^1\) | Diacetyl and/or acetoin\(^1\) | Proteolytic activity as c(FAA)/(mmol/L) | \(A_{560 \text{ nm}}\) | Growth w(NaCl)=2 %\(^3\) | Growth w(NaCl)=4 %\(^3\) |
|---------|------------------|------------|-------------|-------------|-----|------------------------|----------------|------------------|-----------------------------------|-----------|-------------------|-------------------|
| GS1     | cow's milk cheese (Trancas) | (6.60±0.01)\(^a\) | (6.42±0.01)\(^a\) | (6.27±0.02)\(^a\) | 0.33 | 0.0138                | -              | +                | (2.38±0.21)\(^c\) | (0.61±0.23)\(^c\) | 78                | 75                |
| GS9     | cow's milk cheese (Tafi Viejo) | (6.60±0.01)\(^a\) | (5.10±0.03)\(^a\) | (4.69±0.02)\(^c\) | 1.91 | 0.0796                | +              | -                | (3.39±0.25)\(^c\) | (1.10±0.28)\(^bc\) | 56                | 43                |
| GS21    | goat's milk cheese (Trancas) | (6.60±0.01)\(^a\) | (5.85±0.04)\(^b\) | (4.71±0.03)\(^c\) | 1.89 | 0.0788                | +              | +                | (3.34±0.28)\(^c\) | (2.41±0.32)\(^a\) | 60                | 30                |
| GS25    | spicy cheese (Tafi del Valle) | (6.60±0.01)\(^a\) | (5.27±0.01)\(^a\) | (4.01±0.02)\(^c\) | 2.59 | 0.1079                | +              | +++              | (3.84±0.31)\(^ab\) | (1.22±0.24)\(^bc\) | 106               | 83                |
| GS31    | black olives (central market) | (6.60±0.01)\(^a\) | (5.34±0.03)\(^d\) | (4.03±0.02)\(^c\) | 2.57 | 0.1071                | +              | ++               | (1.44±0.19)\(^a\) | (2.78±0.33)\(^a\) | 62                | 23                |
| GS34    | black olives (central market) | (6.60±0.01)\(^a\) | (5.25±0.03)\(^d\) | (4.07±0.04)\(^c\) | 2.53 | 0.1054                | +              | ++               | (0.70±0.13)\(^a\) | (2.09±0.29)\(^ab\) | 85                | 51                |
| GS35    | Pategras cheese (Trancas) | (6.60±0.01)\(^a\) | (5.26±0.02)\(^d\) | (4.12±0.03)\(^c\) | 2.48 | 0.1033                | +              | ++               | (3.05±0.22)\(^cd\) | (0.92±0.14)\(^c\) | 155               | 96                |
| GS43    | cheese with oregano (Tafi del Valle) | (6.60±0.01)\(^a\) | (5.63±0.04)\(^c\) | (3.97±0.02)\(^a\) | 2.63 | 0.1096                | +              | ++               | (4.37±0.25)\(^a\) | (2.62±0.33)\(^a\) | 66                | 33                |
| GS53    | cow's milk cheese (Trancas) | (6.60±0.01)\(^a\) | (5.60±0.06)\(^c\) | (4.75±0.02)\(^c\) | 1.85 | 0.0771                | +              | ++               | (2.73±0.21)\(^d\) | (0.81±0.18)\(^c\) | 81                | 66                |
| GS83    | canned peppers (central market) | (6.60±0.01)\(^a\) | (5.91±0.01)\(^b\) | (4.70±0.03)\(^c\) | 1.90 | 0.0792                | +              | +                | (0.97±0.12)\(^a\) | (1.23±0.28)\(^bc\) | 81                | 60                |
| GS101   | pickles (central market) | (6.60±0.01)\(^a\) | (5.88±0.01)\(^b\) | (4.82±0.02)\(^b\) | 1.78 | 0.0742                | +              | +                | (0.98±0.18)\(^a\) | (1.20±0.23)\(^bc\) | 84                | 47                |
| GS109   | pickles (Concepción) | (6.60±0.01)\(^a\) | (5.34±0.02)\(^d\) | (4.03±0.04)\(^c\) | 2.57 | 0.1071                | +              | ++               | (1.44±0.28)\(^a\) | (2.78±0.38)\(^a\) | 66                | 32                |
| GS110   | pickles (Concepción) | (6.60±0.01)\(^a\) | (5.25±0.02)\(^a\) | (4.07±0.06)\(^a\) | 2.53 | 0.1054                | +              | ++               | (1.70±0.23)\(^d\) | (2.09±0.29)\(^ab\) | 65                | 33                |

Values are average from three independent assays±standard deviation. Mean values with different letters in superscript in the same column indicate significant differences (\(p<0.05\)). ΔpH = \(pH_{24\text{ h\ incubation}}-pH_{\text{initial}}\);\(^1\) + indicates positive milk coagulation, – indicates no coagulation;\(^2\) + indicates low production, ++ medium production, +++ high production and – no production;\(^3\) percentage of \(A_{560 \text{ nm}}\) obtained at the end of growth relative to the control culture developed in a medium without NaCl, FAA=free amino acids.
Some enzyme activities play a key role in fermentation through the release of flavour and bioactive compounds from carbohydrates, lipids and proteins. None of the strains isolated in this study showed amylolytic or lipolytic activities (data not shown), whereas proteolytic activities as FAA varied widely among isolates from (0.70±0.13) to (4.37±0.25) mmol/L. Almost 60 % of dairy isolates showed high proteolytic activities (FAA more than 2 mmol/L) whereas all strains from fermented plants displayed low (FAA less than 1 mmol/L) or intermediate activities (FAA 1 to 2 mmol/L). This distribution could be considered expected taking into account the nature of the food matrices from which both groups of microorganisms were isolated. Milk contains casein as the main protein source and only LAB with efficient proteolytic systems (cell wall proteases and different peptidases) will be able to grow. In contrast, vegetables commonly used for pickles contain low-protein and high mineral and vitamin contents. As in our study, other studies have assessed the proteolytic activities of vegetable LAB to select starter cultures for legume sourdough (21,29) and pickled vegetables (30).

From an industrial point of view, both the acidification capacity and proteolytic activity are preferable features of LAB since they contribute to organoleptic properties and preservation of fermented foods. Additionally, the proteolytic/peptidolytic enzymes of LAB may be involved in the degradation of allergenic and antinutritional proteins and the release of bioactive peptides that are thought to promote health beyond basic nutrition (30). In the dairy industry, acidification activity is indicative of the quality of the LAB starters, but their proteolytic systems play a key role enabling their growth in milk and ensuring a successful fermentation. In addition, proteolytic enzymes release from casein the peptides and amino acids which are involved in the formation of the aroma, texture and flavour of the end product. Regarding fermented plants, the relevance of the acidifying or proteolytic abilities of LAB depends on the nature of the food matrix. For fermented pickles, highly acidifying strains will contribute to the sensory quality and safety of the product, whereas fermented cereals and legumes will require highly proteolytic LAB to allow for their own development, elimination of antinutritional factors, and the increase of protein digestibility. Although the isolates of our study varied in their acidification and proteolytic capacities, the dairy LAB were best adapted to milk environments, since most of them acidified and coagulated milk faster and produced more FAA than strains from fermented plants. Isolates GS25 and GS43 were the most proteolytic and released (3.84±0.31) and (4.37±0.25) mmol/L of FAA, respectively.

Regarding diacetyl and/or acetoin production, 73 % of dairy isolates (25 out of 34 strains) were able to produce different amounts of these flavour compounds, whereas 46 % of LAB (14 out of 30) isolated from fermented plants had this property (Table 1).

Osmotolerance is another important technological property of strains intended for starter cultures of pickled vegetables since NaCl is a key additive in the manufacturing of this kind of food. In the presence of 2 % NaCl most of the isolates (44 out of 64) were able to reach more than 60 % of the final biomass obtained in the absence of salt. Some isolates of dairy origin, such as GS25 and GS35 grew even more than the control (106 and 155 % respectively), while GS34 and GS101, among the plant isolates, reached a biomass close to 85 % of their respective control (Table 2). When culture media contained 4 % NaCl, and 32 or 10 % of dairy and plant LAB, respectively, the growth exceeded 50 % of control biomass (Table 1). The ability to develop under increasing salt mass fractions has served as a selection criterion in different studies. LAB from fermented stink beans were strongly affected by NaCl mass fractions higher than 5 %, while most L. plantarum isolated from fermented vegetables in Turkey were able to tolerate 8 % NaCl (27,30). In the present study, dairy isolates GS25 and GS35 were the most tolerant to 4 % NaCl (83 and 96 % of control biomass), while GS34 and GS83 were the only isolates of plant origin that reached 51 and 60 % of control biomass.

Although strains could be selected by their individual technological features, the presence of various properties concurrently would be more appropriate for the design and development of foods with better sensory properties. Therefore, a principal component analysis (PCA) based on the technological property data of all LAB isolates, regardless of their origin, was performed in order to find the isolates with the best features for the manufacture of fermented vegetables. The estimated parameters analysed by means of PCA revealed two eigenvalues higher than 1 and the first two principal components (PCs) explaining 73.66 % of the total variation, with the first discriminant function (PC1) as the most important, accounting for 52.36 % of the explained variance, while the second function (PC2) explained 21.30 %. Fig. 1 represents the variables and observations projected in the space of PC1 and PC2. Plotting data in a space defined in this way provides a rapid means of visualizing similarities or differences in a data set, allowing improved discrimination among samples or individuals. The study of the contribution of variables to factors, as well as the squared cosines of the variables, showed that factor 1 described mainly acidification capacity of the strains, whereas factor 2 described mainly tolerance to 2 and 4 % NaCl. Osmotolerance variables had a low correlation with acidification properties. The homogenous dispersion of the isolates over the four quadrants evidenced a wide variability in the analysed variables, and the heterogeneity of LAB populations associated with these regional fermented products. The biplot graph led to the identification of a defined group of six LAB that presented simultaneously the best technological properties. This group underwent further identification and characterization.
Genotypic identification of selected LAB

Selected LAB isolates GS31 and GS34 (isolated from pickles and olives) and GS21, GS25, GS35 and GS43 (obtained from artisanal cheese) were taxonomically identified by comparing their 16S rRNA gene sequences with sequences present in the GenBank (17) database and the cured database Ribosomal Database Project (18). Strains GS21 and GS43 showed 99 % identity with Lactobacillus rhamnosus species, whereas strains GS31 and GS34 were 98 and 97 % identical, respectively, to Lactobacillus plantarum. Finally, isolates GS25 and GS35 were identified as Weissella viridescens (100 % identity) and Weissella paramesenteroides (99 % identity) respectively. The strains were deposited in the Microbial Culture Collection of CERELA, Tucumán, Argentina (affiliated to the World Federation for Culture Collections) and renamed with the code numbers provided by the Collection (Table 3).

Other studies have reported the presence of the same species of non-starter LAB on spontaneously fermented food of different origins and natures. L. plantarum occurs with high frequency in different fermented vegetables around the world such as brined olives (28,31), sauerkraut (32), and pickles (4,5,26,30). L. rhamnosus niches are more diverse, with probiotic strains originating from the human intestine (33) and other relevant isolates obtained from cheese ripening (34). Weissella strains have been isolated on the surface of fruits, beans, fermented vegetables and juices, and in some dairy products (21,32,35).

Tannase and gallate decarboxylase activities of selected LAB

In addition to metabolic activities relevant for the organoleptic properties of the product, other features, such as the presence of enzymes involved in the removal of ANF present in the food matrix and/or the generation of bioactive metabolites could be relevant for the selection of strains as starter cultures. Vegetable tannins, present in large quantities in many plants used as food and feed, are considered nutritionally undesirable since they inhibit digestive enzymes and affect the utilization

Table 3. Identification by 16S rRNA gene sequence of lactic acid bacteria selected based on the presence of enzymes involved in antinutritional factors, reduction/bioactive compound generation and pathogen inhibition

| Isolate | Closest relative         | Identity/% | CRL code | Accession number | Tannase | Gallate decarboxylase | Pathogen inhibition by cell-free supernatant | BLIS production |
|---------|--------------------------|------------|----------|------------------|---------|----------------------|---------------------------------------------|----------------|
| GS21    | Lactobacillus rhamnosus  | 99         | 2159     | LT714201         | –       | –                    | +                                           | –              |
| GS25    | Weissella viridescens    | 100        | 2160     | LT714202         | –       | –                    | +                                           | –              |
| GS31    | Lactobacillus plantarum  | 98         | 2161     | LT714203         | +       | –                    | +                                           | –              |
| GS34    | Lactobacillus plantarum  | 97         | 2162     | LT714204         | +       | +                    | –                                           | –              |
| GS35    | Weissella paramesenteroides | 99     | 2163     | LT714205         | –       | +                    | –                                           | –              |
| GS43    | Lactobacillus rhamnosus  | 99         | 2164     | LT714206         | –       | –                    | +                                           | –              |

BLIS=bacteriocin-like inhibitory substance
of vitamins and minerals. Tannase releases gallic acid from hydrolysable tannins whereas gallate decarboxylase decarboxylates gallic acid to pyrogallol (36). Both enzymes could be relevant for the removal of tannins and the release of bioactive phenolic compounds. In the present study, both Lactobacillus plantarum CRL2161 and CRL2162 were positive for tannase and gallate decarboxylase activities, whereas Weissella paramesenteroides CRL2163 displayed only gallate decarboxylase activity (Table 3). The presence of these enzymes has been reported previously in strains of L. plantarum (36) and other LAB species such as L. pentosus and L. paraplanatarum (20). Our group has recently identified gallate decarboxylase activity in Weissella species (21). Description of degradation of complex hydrolysable tannins and phenolic acids such as gallic acid by L. plantarum, L. brevis, as well as by other microbes from herbivore guts such as Enterococcus faecalis and Streptococcus galloyticus among others are available (37–40).

**Growth of selected LAB under saline and acidic conditions**

The submergence of vegetables in brine with or without the addition of vinegar represents the most characteristic step of pickle manufacturing. The purpose of the addition of NaCl is to enhance flavour and to prevent the growth of harmful microorganisms. Since vegetables remain exposed to saline throughout the shelf life of the product, the ability of bacteria to grow and remain viable in this environment is highly desirable. Therefore, we assessed the ability of selected LAB to grow under increasing concentrations of NaCl. The kinetics of growth determined by absorbance and growth parameters obtained by applying the Gompertz model are presented in Fig. 2 and Table 4, respectively. As a general observation, the experimental data of the growth of each LAB under the different conditions assayed showed a high goodness of fit to the model ($R^2 > 0.97$), in agreement with other studies (12, 41).

Regarding growth parameters, as was expected, increasing the NaCl concentration had a negative effect on the growth of all strains, with subsequent decreases in maximum growth rates ($\mu$) and maximum absorbance ($A_t$) reached at the end of incubation, and increases of lag phase duration ($t_g$) and generation time ($t_\sigma$).

At 2% NaCl, all strains were able to reach more than 70% of control biomass without salt, whereas when grown in 4% NaCl, only four strains attained more than 50% of the control growth. Strains L. plantarum CRL2162 and L. rhamnosus CRL2164 showed better growth parameters than the other selected strains ($A_\sigma = 0.989$ and 0.982, $\mu = 0.105$ and 0.107 h$^{-1}$, and $t_\sigma = 2.857$ and 2.794 h, respectively) (Table 4). When the NaCl mass fraction in the medium was 7%, W. viridescens CRL2160 and L. plantarum CRL2161 reached less than 10% of control growth, while the rest of the LAB were able to reach up to 30% of their control biomass. Finally, at the highest salt mass fraction assayed (10% NaCl) none of the tested strains was able to grow. The observed behaviour was better than that reported by Pundir et al. (42), who determined that their vegetable-derived strains could grow in up to 6.5% NaCl; however, that was worse than the performance of L. plantarum strains isolated from fermented vegetables in Turkey that were able to tolerate 9% NaCl (30).

In the same manner, proliferation of LAB under acidic conditions represents a valuable feature as it contributes to acidity and biopreservation of the product, and prolongs their ability to provide desirable characteristics to the product and/or benefits to the consumer. Fig. 3 and Table 5 show growth curves and parameters obtained by Gompertz model (12). As observed with osmotolerance, the increase in acidity of the growth media negatively affected growth parameters in most cases. At pH = 5.5, L. rhamnosus CRL2164 showed better parameters than those observed in the control culture at pH = 7 ($A_\sigma = 2.793$, $\mu = 0.206$ h$^{-1}$, $t_\sigma = 2.202$ h) vs $A_\sigma = 2.114$, $\mu = 0.136$ h$^{-1}$, $t_\sigma = 2.202$ h), whereas L. plantarum CRL2162 showed similar growth to its respective control culture. At lower pH values (5.0 to 4.0), these two strains were also the best adapted to stressful conditions (in agreement with osmotolerance), since their growth behaviour was less affected than the other selected LAB. Weissella strains (CRL2160 and CRL2163) were the most sensitive to acid and were unable to grow at pH = 4.0.

Yu et al. (4) characterized the lactic microflora present in Chinese pickles and observed that all isolates identified as L. alimentarius and L. plantarum were able to develop at pH = 4.0 and 4.5, and in 6.0 and 6.5% NaCl, supporting the frequent selection of these species as starter cultures for this type of fermented food.

![Fig. 2. Growth kinetics of most osmotolerant lactic acid bacteria developed in LAPtg broth containing 2, 4, 7 and 10% NaCl. Absorbance measured at 560 nm determined the biomass after 24 h of incubation at 37° C. Panel: a) L. plantarum CRL2162, and b) L. rhamnosus CRL2164. Results are the mean value of three independent trials±standard deviation](image-url)
Table 4. Growth parameters of selected lactic acid bacteria at different NaCl mass fractions estimated with mathematical model of Gompertz (12)

| Strain   | Parameter | Control | 2       | 4       | 7       | 10      |
|----------|-----------|---------|---------|---------|---------|---------|
|          |           |         |         |         |         |         |
| CRL2159  | A         | 2.026   | 1.422   | 0.586   | 0.514   | 0.098   |
|          | µ/h⁻¹     | 0.170   | 0.108   | 0.074   | 0.064   | 0.016   |
|          | t/µL      | 2.63    | 1.574   | 1.701   | 2.644   | 0.283   |
|          | tₚ/µL     | 1.773   | 2.772   | 4.046   | 4.681   | 18.684  |
|          | R         | 0.991   | 0.997   | 0.998   | 0.997   | 0.975   |
|          | v/µL      | 98      | 99      | 98      | 99      | 95      |
| CRL2160  | A         | 0.789   | 0.796   | 0.708   |         |         |
|          | µ/h⁻¹     | 0.061   | 0.059   | 0.054   |         |         |
|          | t/µL      | 0.131   | 0.769   | 0.791   |         |         |
|          | tₚ/µL     | 4.930   | 5.071   | 5.480   |         |         |
|          | R         | 0.991   | 0.994   | 0.990   |         |         |
|          | v/µL      | 98      | 98      | 98      |         |         |
| CRL2161  | A         | 2.012   | 1.796   | 0.645   |         |         |
|          | µ/h⁻¹     | 0.152   | 0.130   | 0.073   |         |         |
|          | t/µL      | 1.865   | 1.742   | 0.871   |         |         |
|          | tₚ/µL     | 1.974   | 2.304   | 4.085   |         |         |
|          | R         | 0.997   | 0.993   | 0.997   |         |         |
|          | v/µL      | 99      | 98      | 99      |         |         |
| CRL2162  | A         | 1.655   | 1.511   | 0.989   | 0.518   |         |
|          | µ/h⁻¹     | 0.132   | 0.121   | 0.105   | 0.084   |         |
|          | t/µL      | 1.108   | 1.014   | 1.100   | 1.969   |         |
|          | tₚ/µL     | 2.268   | 2.485   | 2.857   | 3.556   |         |
|          | R         | 0.996   | 0.994   | 0.996   | 0.996   |         |
|          | v/µL      | 99      | 98      | 99      | 99      |         |
| CRL2163  | A         | 0.638   | 0.494   | 0.449   | 0.162   |         |
|          | µ/h⁻¹     | 0.059   | 0.058   | 0.044   | 0.024   |         |
|          | t/µL      | 0.680   | 0.381   | 1.687   | 0.370   |         |
|          | tₚ/µL     | 5.099   | 5.160   | 6.816   | 12.096  |         |
|          | R         | 0.999   | 0.995   | 0.996   | 0.998   |         |
|          | v/µL      | 98      | 99      | 99      | 99      |         |
| CRL2164  | A         | 2.114   | 1.861   | 0.982   | 0.570   |         |
|          | µ/h⁻¹     | 0.136   | 0.133   | 0.107   | 0.051   |         |
|          | t/µL      | 1.174   | 1.406   | 1.312   | 1.226   |         |
|          | tₚ/µL     | 2.202   | 2.260   | 2.794   | 5.812   |         |
|          | R         | 0.995   | 0.997   | 0.993   | 0.997   |         |
|          | v/µL      | 99      | 99      | 98      | 99      |         |

A= increase of absorbance between A₀ and Aₙ, µ=max growth rate, tₚ=duration time of lag phase, tₚ=generation time (doubling time of the bacterial population), R= Pearson’s coefficient of determination, v=coefficient of correlation, – indicates no growth of the microorganism

Fig. 3. Growth kinetics of most osmotolerant lactic acid bacteria developed in LAPTg broth adjusted to pH=4.0, 4.5, 5.0 and 5.5. Absorbance measured at 560 nm determined the biomass after 24 h of incubation at 37 °C. Panel: a) L. plantarum CRL2162, and b) L. rhamnosus CRL2164. Results are the mean value of three independent trials±standard deviation
Antimicrobial activity of selected LAB

Uncontrolled spontaneous vegetable fermentations are frequently sensitive to contamination from spoilage and pathogenic microorganisms, which may represent a risk to public health. It is well-known that LAB produce several antimicrobials (organic acids, H₂O₂, ethanol and bacteriocins), which can be used in food processing as alternative to chemical preservatives (43). In the present study, the ability of the selected strains to inhibit the growth of *Escherichia coli* and *Listeria innocua*, two foodborne species strongly related to the consumption of vegetable foods, was assessed as a safety property. Non-neutralized supernatants of all strains inhibited growth of *E. coli*, whereas only three of them (*L. plantarum* CRL2161, CRL2162 and *L. rhamnosus* CRL2164) were able to inhibit *Listeria innocua* (Table 3). When testing neutralized supernatants, there was no antagonistic activity of any LAB against the two assayed pathogens, suggesting the absence of other inhibitory substances such as H₂O₂ or bacteriocins besides organic acids.

Other studies have demonstrated the ability of *L. plantarum* and other lactobacilli from fermented vegetables to inhibit foodborne pathogens. Çon and Karasu (28) observed that all strains of *L. plantarum* isolated from olives and pickles from western Turkey were able to inhibit *L. monocytogenes* and *E. coli* by lowering the pH, and to a lesser extent other pathogens. Rao et al. (44) found that *L. plantarum* E11, isolated from Chinese pickles from the fifth day of pickling

Table 5. Growth parameters of selected lactic acid bacteria at different pH estimated with mathematical model of Gompertz (12)

| Strain    | Parameter | Control | pH  |
|-----------|-----------|---------|-----|
|           |           |         | 5.5 | 5.0 | 4.5 | 4.0 |
| CRL2159   | A         | 2.026   | 1.677| 1.814| 1.353| 0.512|
|           | μ/h⁻¹     | 0.170   | 0.111| 0.103| 0.094| 0.046|
|           | t₀/h      | 2.63    | 2.405| 2.223| 3.737| 0.960|
|           | tₚ/h      | 1.773   | 2.703| 2.914| 3.194| 6.489|
|           | R         | 0.991   | 0.997| 0.993| 0.983| 0.995|
|           | ν%        | 98      | 99   | 98   | 96   | 99   |
| CRL2160   | A         | 0.789   | 0.757| 0.521|       |     |
|           | μ/h⁻¹     | 0.061   | 0.060| 0.032|       |     |
|           | t₀/h      | 0.131   | 1.138| 1.431|       |     |
|           | tₚ/h      | 4.930   | 4.982| 9.348|       |     |
|           | R         | 0.991   | 0.995| 0.996|       |     |
|           | ν%        | 98      | 99   | 99   |       |     |
| CRL2161   | A         | 2.021   | 1.635| 1.242| 1.274| 0.644|
|           | μ/h⁻¹     | 0.152   | 0.125| 0.108| 0.097| 0.057|
|           | t₀/h      | 1.865   | 1.985| 2.069| 2.236| 1.789|
|           | tₚ/h      | 1.974   | 2.392| 2.764| 3.095| 5.228|
|           | R         | 0.997   | 0.993| 0.998| 0.999| 0.995|
|           | ν%        | 99      | 98   | 99   | 99   | 99   |
| CRL2162   | A         | 1.655   | 1.535| 1.686| 1.405| 0.226|
|           | μ/h⁻¹     | 0.132   | 0.132| 0.128| 0.092| 0.027|
|           | t₀/h      | 1.108   | 1.593| 1.507| 1.184| 0.275|
|           | tₚ/h      | 2.268   | 2.277| 2.347| 3.262| 10.944|
|           | R         | 0.996   | 0.992| 0.993| 0.997| 0.998|
|           | ν%        | 99      | 98   | 99   | 99   | 99   |
| CRL2163   | A         | 0.638   | 0.705| 0.554| 0.377|       |
|           | μ/h⁻¹     | 0.059   | 0.057| 0.046| 0.026|       |
|           | t₀/h      | 0.680   | 0.818| 1.751| 0.743|       |
|           | tₚ/h      | 5.099   | 5.193| 6.500| 11.489|       |
|           | R         | 0.999   | 0.993| 0.997| 0.998|       |
|           | ν%        | 98      | 98   | 99   | 99   | 99   |
| CRL2164   | A         | 2.114   | 2.793| 1.749| 1.653| 0.754|
|           | μ/h⁻¹     | 0.136   | 0.206| 0.155| 0.119| 0.074|
|           | t₀/h      | 1.174   | 2.669| 1.690| 1.298| 0.769|
|           | tₚ/h      | 2.202   | 1.458| 1.933| 2.523| 4.029|
|           | R         | 0.995   | 0.992| 0.995| 0.996| 0.996|
|           | ν%        | 99      | 99   | 99   | 99   | 99   |

A= increase of absorbance between A₀ and Aₙ; μ= maximum growth rate; t₀= duration of lag phase; tₚ= generation time (doubling time of the bacterial population); R= Pearson’s coefficient of determination; ν= coefficient of correlation; – indicates no growth of the microorganism.
fermentation, produced a bacteriocin-like substance that inhibited *E. coli*, *L. monocytogenes* and *Staphylococcus aureus*, prolonging the safety and shelf life of this food.

**Compatibility of strains**

Starter cultures may consist of a single strain (monostrain) or several strains (multistrain) of the same or different species, which are combined to look for a synergism in the fermentation process (45). When evaluating potential multistrain starter cultures, it is important to carry out compatibility tests in order to avoid the combining of strains showing inhibitory activity against each other, either through the production of inhibitory substances, competition for nutrients or intercellular interactions (22). In the present study, the compatibility of different LAB was determined by inhibition of growth with an agar diffusion assay and a cross-streak assay. The first method served to assess the production of antagonistic substances, whereas the second method assessed inhibition by direct interaction of microorganisms grown on the surface of an agar medium.

No inhibition halos of selected LAB cell-free supernatants against the other strains was observable, suggesting the absence of antimicrobial substances that could inhibit the development of strains combined in mixed cultures. The cross-streak assay showed similar results, as no evident competition was noticeable at sites of co-growth in solid medium in any combination of LAB assayed, allowing their potential use as a multistrain starter (results not shown).

**CONCLUSIONS**

In the current study we isolated, technologically characterized and selected LAB for the development of autochthonous starter cultures for the pickling industry. Six LAB isolates belonging to the *Lactobacillus plantarum*, *L. rhamnosus*, *Weissella paramesenteroides* and *W. viridicens* species showed promising properties. No direct antagonism was observable between strains that could dismiss their use as mixed cultures. *L. plantarum* CRL2162 and *L. rhamnosus* CRL2164 performed the best under pickling conditions with high growth and inhibitory activity against *Escherichia coli* and *Listeria innocua*, so they should be the most appropriate for inoculation and fermentation of vegetable foods. Further studies regarding their most suitable LAB ratios and their application in vegetable pickle manufacturing at laboratory and pilot scale are presently ongoing. From an industrial point of view, the development of a new starter culture will allow for the obtaining of standardized fermented vegetable products of high quality, increasing the competitiveness of Argentinian production.

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**CONFLICTS OF INTEREST**

The authors declare no conflicts of interest.

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