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Isolation and selection of potential probiotic lactic acid bacteria from *Opuntia ficus-indica* fruits that grow in Northwest Argentina

Hernán E. Verón [a], H. Daniel Di Risio [b], María Inés Isla [a, b], Sebastian Torres [a, b, *]

[a] Laboratorio de Investigación de Productos Naturales (LIPRON), Instituto de Bioprospección y Fisiología Vegetal (INBIOFIV-CONICET), San Miguel de Tucumán, Tucumán, Argentina
[b] Facultad de Ciencias Naturales e IML, Universidad Nacional de Tucumán, San Miguel de Tucumán, Tucumán, Argentina

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ABSTRACT

Lactic acid fermentation of cactus fruit (*Opuntia ficus-indica*) juice constitutes an important biotechnology process for its preservation. The present study shows the isolation and selection of potential probiotic autochthonous strains for the preparation of a fermented cactus fruit juice. 17 strains of lactic acid bacteria were isolated from *O. ficus-indica* fruits that grow in arid regions from Argentina. Isolates were screened for probiotic traits such as gastrointestinal stress tolerance, cell surface properties and antimicrobial activity, and also for their effects on functional properties of fermented juices. Among 17 isolates, 4 showed singular properties to be used as starters in cactus pear juice fermentation. These strains were identified as *Lactobacillus plantarum* S-811, *L. plantarum* S-TF2, *Fructobacillus fructosus* S-22, and *F. fructosus* S-TF7. The selected strains have potential for their use in the fermentation of cactus pear juice contributing to its preservation and the conservation of its health-promoting features.

1. Introduction

Scientific evidence has demonstrated the benefits from fruits and vegetables ingestion with special consideration about its potentially bioactive constituents, which may be protective against oxidative damage (Butera et al., 2002; Livrea & Tesoriere, 2006). Many studies have indeed shown the positive relation between consumption of a diet rich in fruits and vegetables, and reducing the risks of some age-related pathologies, including cancer, cardiovascular diseases, neurodegenerative disorders or diabetes (Livrea & Tesoriere, 2006; Panda et al., 2017). In this context, the cactus pear fruit of *Opuntia ficus-indica* have attracted great interest because of their nutritional and health-promoting properties (Moβhammer, Stintzing, & Carle, 2006; Panda et al., 2017). Cactus pear fruits are a significant source of sugars (e.g., glucose and fructose), minerals (e.g., calcium and magnesium), prebiotic fiber, and numerous antioxidant compounds, such as vitamin C, tocopherols, carotenoids, flavonoids (e.g., rutin, kaempferol, quercetin, isorhamnetin, and its derivatives), phenolic acids, biothiols (e.g., reduced glutathione, cysteine, and n-acetyl cysteine), the cell-protective amino acid Taurine, and betalain pigments (e.g., betaxanthins and betacyanins) (Livrea & Tesoriere, 2006; Moβhammer et al., 2006; Tesoriere et al., 2012; Panda et al., 2017). The nutraceutical benefits of this fruit are believed to mainly stem from its recognized antioxidant properties, which are related to the above functional compounds (Coria Cayupán, Ochoa, & Nazareno, 2011; Stintzing et al., 2005). Indeed, there is sufficient scientific evidence supporting the anti-inflammatory, anticancerigenic, antiulcerogenic, hypocholesterolemic, hypoglycemic, hepatoprotective, and immunestimulatory properties of these healthful components of cactus pear fruit (Coria Cayupán et al., 2011; Jiménez-Aguilar et al., 2015; Moβhammer et al., 2006; Panda et al., 2017; Stintzing et al., 2005; Tesoriere et al., 2012).

Cactus pears are native to tropical America, but they can be found in sub-tropical and tropical areas worldwide (Cassano, Conidi, Timpone, D'Avella, & Drioli, 2007). Cactus pear plants are suitable to cultivate in arid and semiarid regions from Argentina, making this crop an interesting agricultural resource for local inhabitants. The fruit is a fleshy berry with a number of hard seeds, varying in shape, size and colour. Cactus pear is usually consumed in Argentina as a fresh fruit, juices, jams, marmalades, *arrope* and alcoholic beverages (Nazareno & Padrón Pereira, 2011; Coria Cayupán et al., 2011). The rather high sugar content and low acidity (pH > 4.5) of the fruit give it a delicious, sweet taste (Cassano et al., 2007). Unfortunately, these positive sensory characteristics make this fruit susceptible to microbial attack limiting its storage life. The increasing market demand for this fruit has challenged researchers to develop process to lengthen storage life. Regarding

* Corresponding author. INBIOFIV- CONICET, Universidad Nacional de Tucumán, San Lorenzo 1469, 4000, San Miguel de Tucumán, Argentina.
E-mail address: sebatk@hotmail.com (S. Torres).

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the juice, different procedures were developed to prevent spoilage (Cassano et al., 2007; Moβhammer et al., 2006; Saenz, 2000). Although these procedures guarantee microbiological stability, the final products do not resemble to original fresh juice due to changes in colour, flavour and nutritional value, and also decrease of health-promoting properties (Moβhammer et al., 2006). In this sense, lactic acid fermentation of vegetables, currently used for the bio-preservation of various foods, emerges as an important biotechnology for maintaining or improving safety, nutritional, sensory and shelf-life properties of vegetables and by-products thereof (Di Cagno, Coda, De Angelis, & Gobbetti, 2013).

Lactic acid bacteria are a small part of the autochthonous microbiota of vegetables and fruits (Di Cagno et al., 2013). Since each type of plant provides a unique niche due to the availability of nutrients that provides and the competitive microbiota that harbours, it is assumed that each species of plant has a dominant microbiota (Di Cagno et al., 2013). The main species isolated from vegetables and fruits belong to *Leuconostoc*, *Lactobacillus*, *Weissella*, *Enterococcus* and *Pediococcus* genera, being *Lactobacillus plantarum* one of the most frequently isolated species. Many of these bacteria can ferment plant spontaneously, however, the risk of fermentation failure, in terms of inadequate inhibition of spoilage and pathogen microorganisms, and undesired sensory and nutritional properties, make a controlled fermentation, using selected starters, often preferred (Di Cagno et al., 2013). In this regard, selection of autochthonous starters from vegetables and fruits is recommended. Autochthonous cultures are adapted for the specific plant matrix and therefore may ensure better performance (prolonged shelf life and specific nutritional and sensory properties) compared to allochthonous strains (Di Cagno et al., 2013; Vera-Pingitore et al., 2016). Many of these autochthonous lactic acid bacteria strains isolated from fruits and vegetables and used as starters in plant-based fermented products are probiotics (Vitali et al., 2012). Probiotics are live microorganisms that benefit the health of the host when ingested in sufficient amounts (FAO & WHO, 2006). Potential health-promoting effects of probiotics mainly rely on their ability to improve nutritional and gut microbiota balance, as well as their capacity to modulate mucosal and systemic immunity (Todorov, LeBlanc, Bernadette, & Franco, 2012). These beneficial effects depend on the ability of probiotic strains to survive the human gastrointestinal tract conditions (gastric acidity, digestive enzymes, bile salts) (Vitali et al., 2012). Furthermore, other several functional features are expected in probiotic strains, such as the capacity of adhesion to mucosal surfaces, the antagonistic activity against pathogenic and spoilage microorganisms or functional properties like feruloyl esterase activity, enzyme required to improve the bioavailability of ferulic acid and other phenolics in the intestine (Abeijon Mukdsi, Haro, Gonzalez, & Medina, 2013; Vitali et al., 2012). In a recent work, autochthonous strains of *Leuc. mesenteroides* were identified and used for fermentation of cactus pear puree (Di Cagno et al., 2016). However, to our knowledge, no researches have evaluated the use of probiotic autochthonous lactic acid bacteria for the fermentation of cactus pear juices. This work describes the isolation and selection of potential probiotic strains of autochthonous lactic acid bacteria candidate for the preparation of a fermented beverage from *O. ficus-indica* fruit juice.

2. Materials and methods

2.1. Fruit samples

O. ficus-indica cactus pears of the “green cultivar” were aseptically collected in the northwest of the province of Tucumán (Colalao del Valle, Tucuman, Argentina). Fruits were harvested during February, corresponding to the summer season, when characteristic ripe skin colour became manifest. Hand-picked fruits were separated into two batches, one of which was washed in water, weighed, frozen and stored at −20 °C. The other batch that was used for microorganism isolation, was aseptically stored at 4 °C without washed.

2.2. Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from fresh and spoiled cactus pear fruit. Briefly: Ten grams of cactus pear fruit were suspended in 90 ml of sterile sodium chloride (0.9%, w/v) solution, disrupted and homogenized for 5 min at room temperature. Serial dilutions were made and 0.1 ml aliquots were plated onto MRS agar (Oxoid Ltd., Basingstoke, Hampshire, England), followed by incubation at 37 °C for 48 h under microaerophilic conditions, for isolating presumptive mesophilic lactic acid bacteria. Colonies apparently with different morphology were randomly picked from the MRS agar plates of the highest dilution for isolation. The isolated strains were purified by streaking on MRS agar before being subjected to characterization.

The isolates were Gram-stained and tested for catalase reaction and motility. Presumptive LAB were selected based on the morphology and then, were characterized by their growth at various temperatures (15, 42, 45 °C), production of gas from glucose, capacity of acidifying the culture medium. Isolates were stored in MRS or MH broth (Oxoid) with 20% (v/v) glycerol at −20 °C.

2.3. Preparation of cactus pear juice

Juice from cactus pears was produced as follows. One (1) kg of fruits were processed to obtain 500 ml of cactus pear juice. Only ripe fruit without blemishes or damage were used to obtain the juice. The fruits were manually peeled and the pulp was homogenized using a blender coupled with a stainless steel strainer (Philips Essence HR1357, China) in order to remove the seeds and mesocarp fibers. The obtained juice was stored at −20 °C until use. Before inoculation of lactic acid bacteria, the juice was defrosted and centrifuged (15,000 × *g*, 20 min) for complete separation of mesocarp fibers and for removal of insoluble material. Then, juice was pasteurized by heating at 64 °C for 30 min and used for the preparation of fermented cactus pear juices.

2.4. Cactus pear juice fermentation

Juice fermentation was performed in 11 ml capacity tubes with screw cap with hermetic seal. Each tube contained 7.5 ml of cactus pear juice, leaving a headspace of 3.5 ml. All fermentation experiences were carried out in triplicate. LAB isolated from *O. ficus-indica* fruits were used as the single autochthonous starter for the fermentation of the juice. Eighty-hour-old (overnight) cells cultivated in MRS broth at 37 °C were harvested by centrifugation (12,000 × *g*, 5 min) and washed twice in sterile saline (0.9% w/v NaCl) solution. Cells were re-suspended to the original volume in sterile saline solution and used to inoculate pasteurized cactus pear juices (2%, v/v), followed by incubation (48 h) at 37 °C. Changes in juices pH values were recorded at 6, 12, 24 and 48 h of incubation. Pasteurized cactus pear juice not inoculated with the LAB and subject to the same treatment was used as the control. Control and fermented juices were stored in the same tubes in which the fermentation was carried out, in a refrigerator (4 °C) for 30 days, and samples were analyzed at 0, 10 and 30 days of storage.

2.5. In vitro probiotic studies

2.5.1. Tolerance to simulated gastric juice

The assay was performed according to Vinderola et al. (2008) with slight modifications. Simulated gastric juice was prepared by dissolving pepsin from porcine gastric mucosa (Sigma) in 0.5% (w/v) sterile saline (NaCl) solution to a final concentration of 0.3% (w/v) and adjusting the pH to 2 and 3. Overnight cultures grown in MRS broth (18 h, 37 °C) were centrifuged at 12,000 × *g* for 5 min, washed twice in sterile saline (0.9% w/v NaCl) solution and re-suspended to the original volume in sterile saline solution. Fifty microliters (50 μl) of washed cell suspension of each strain were mixed with 950 μl of gastric solution pH 2 and 3. After vortexing at maximum setting for 5 s, samples were incubated at 37 °C for 120 min. Sterile saline solution (0.9% w/v) was used as control. Microbial counts (cfu/ml) on MRS agar were determined in controls and gastric solutions after incubation.

2.5.2. Bile tolerance

Bile tolerance was determined according to Vinderola et al. (2008) using the well-diffusion agar assay with slight modifications. Briefly, 20 ml of MRS agar were mixed with 200 μl of overnight cultures of each isolate grown in MRS broth (18 h, 37 °C) and plated in Petri dishes. Wells (5 mm in diameter) were made in the agar layer and 30 μl of a bile salts (Oxoid) solution (0.0, 0.3, 0.6, 1.0 or 2.0 g/100 ml) were placed in each well. Plates were incubated 24 h at 37 °C (microaerophilia) and the diameters of inhibition halos were recorded.

2.5.3. Cell surface hydrophobicity

Hydrophobicity was determined according to the assay of Kos et al. (2003) with some modifications, by measurement of partition of bacterial cells between organic (*p*-xylene) (Cicarelli, Argentina) and aqueous phases. Overnight cultures of LAB grown in MRS broth were harvested by centrifugation at 12000 × *g* for 5 min, washed twice in sterile saline (0.9% w/v NaCl) solution, and finally re-suspended in the same solution. The cell suspension was adjusted to an A_{560nm} value of 0.6 with the saline solution and 500 μl of this suspension were mixed with 500 μl of *p*-xylene during 120 s. The aqueous phase was carefully removed and the A_{560nm} was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\% = [(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance before and after extraction with *p*-xylene, respectively.

2.5.4. Autoaggregation

Autoaggregation assay was carried out according to Montel et al. (2012) with some modifications. Overnight cultures of LAB grown in MRS broth were centrifuged at 12,000 × g for 5 min, and re-suspended in sterile saline solution to obtain an A_{560nm} value of 0.6. These bacterial suspensions (10 ml) were vortexed for 15 s and leave to sediment over 4 h at room temperature. Autoaggregation was determined each 30 min using the formula $A\% = [(A_0 - A_t)/A_0] \times 100$, where A_0 represents the absorbance at time zero and A_t, the absorbance at t time.

2.5.5. Antibacterial activity against cactus pear juice contaminant

The antimicrobial activity of the fermented juices was assessed on a strain of *Bacillus* sp. isolated from decaying cactus pear juice. Antimicrobial activity was tested in fermented juices stored during 10 and 30 days at 4 °C. For this purpose, each fermented juice was sterilized by filtration through a 0.22-μm pore filter (Millipore) before use.

2.5.5.1. Agar diffusion method. Petri dishes containing Mueller Hinton agar (20 ml) were inoculated with 100 μl of an overnight culture of a strain of *Bacillus* sp. previously isolated from decaying juice ($OD_{560nm} = 0.08$). The inoculum was dispersed using a Drigalsky spatula. Wells (7 mm in diameter) were made in the agar layer and the fermented juices with each LAB isolate was placed in each well. Plates were incubated overnight (37 °C, aerobiosis) and the diameters (mm) of the inhibition halos were recorded.

2.5.5.2. Inoculation of Bacillus sp. in fermented juices ("in situ" inhibition). In order to gain further insight of the inhibitory effect of fermented juices on the contaminating microorganism *Bacillus* sp., this bacterium was inoculated in juices that showed inhibitory effect against it in the agar diffusion assay (2.5.5.2). Each fermented juice was filter-sterilized using a 0.22-μm pore size membrane filter (Millipore), and further inoculated with a suspension of *Bacillus* sp. (overnight cultured) in sterile saline to a final OD_{560nm} of 0.08. Then, each juice was incubated at room temperature for 1 h. Then, an aliquot (100 μl) of each juice was dispersed on Mueller Hinton agar and incubated at 37 °C. After 24 and 48 h of growth, the colonies number was counted.

2.5.6. Screening of ferulic acid esterase activity

Screening of feruloyl esterase activity was determined according to Donaghy, Kelly, and McKay (1998) assay. Briefly, 100 μl of overnight cultures (18 h, 37 °C) of each isolate were inoculated on MRS agar, without glucose, supplemented with 1.5% (v/v) of ethyl ferulate. Plates were incubated during 72 h at 37 °C. Feruloyl esterase production was evidenced by the formation of a clearing zone around the zone of inoculation.

2.6. Analysis of bioactive compounds in fermented cactus fruit juices

2.6.1. Determination of total phenolics concentration

Total phenolic compounds content was determined by the Folin-Ciocalteau method according to Singleton, Orthofer, and Lamuela-Raventos (1999). Results were expressed in milligrams of gallic acid equivalents per liter of juice (mg of GAE/l) or per gram of juice dry matter (mg of GAE/g DW). The dry matter of the juices was determined by freeze drying process followed by oven drying at 45 °C until constant weight.

2.6.2. Photometric quantification of betalains

Betalain contents were determined as previously described (Stintzing, Schieber, & Carle, 2003). The juice was diluted with distilled water to obtain absorption values of $0.3 \leq A \leq 0.6$. The betalain content (BC) was calculated by using the following equation: $BC\ (mg/L) = (A \times DF \times MW \times 1000)/(\varepsilon \times L)$; where A is the absorption at 538 and 480 nm for betacyanins and betaxanthins, respectively; DF is the dilution factor and L the pathlength (1-cm) of the cuvette. For quantification of betacyanins and betaxanthins, the molecular weights (MW) and molar extinction coefficients (ε) of betanin (MW = 550 g/mol; ε = 60,000 L/mol cm in H2O; $\lambda = 538$ nm) and indicaxanthin (MW = 308 g/mol; $\varepsilon = 48{,}000$ L/mol cm in H_2O; $\lambda = 480$ nm) were applied.

2.7. Measurement of antioxidant capacity of fermented cactus pear juices

The antioxidant capacity assay was carried out by the improved $ABTS^{\bullet+}$ method as described by Re et al. (1999). Different dilutions of juices (equivalent to 2 to 25 μgGAE/ml total phenolic compounds) were added to $ABTS^{\bullet+}$ solution (1 ml) and mixed thoroughly. Absorbance was recorded at 734 nm, 1 and 6 min after

initial mixing. Results were expressed as SC_{50}, which represents the total phenolics concentration (mg of GAE/l) required to scavenge 50% ABTS cation radical.

2.8. Genotypic identification of selected lactic acid bacteria

Genotypic identification was carried out in most promising LAB strains for cactus pear juice fermentation. Identification of selected LAB with different RAPD-PCR profiles was carried out by partial 16S rRNA gene sequencing. Genomic DNA was extracted as described by Pospiech and Neumann (1995). Oligonucleotide primers (PLB16, 5′-AGAGTTTGATCCTGGCTCAG-3′ and MLB16, 5′-GGCTGCTGGCACG-TAGTTAG-3′) were used to amplify the variable (V1) region of the 16S ribosomal RNA gene. PCR products were electrophoresed in 1.0% (w/v) agarose gels, stained and visualized as above described. Amplicons were excised from the gel and purified using a GFX PCR DNA gel bandpurification kit (GE Healthcare, UK). Purified PCR products were sequenced at CERELA-CONICET by using an ABI 3130 DNA sequencer (Applied Biosystems, Foster, CA). Taxonomic strain identification was performed by comparing the sequences for each isolate with those reported in the Basic BLAST database (NCBI; National Center for Biotechnology Information) and SeqMatch database (RDP; Ribosomal Database Project, Release 11.2). Strains showing homology of at least 97% were considered to belong to the same species.

2.9. Statistical analysis

Results are expressed as the mean ± standard deviation of two or more experiments with duplicate determinations. The statistical analyses were performed in SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The results were analyzed using one-way analysis of variance (ANOVA) and two-way ANOVA. Differences between fermented juices stored for 0, 10 and 30 days with the control (unfermented) juice stored for the same period, and differences between baselines in each juice were verified using one-way ANOVA test, with Tukey's test as a post-hoc comparison of means. The effects of different LAB starters and the storage time on functional parameters of fermented juices were evaluated by the two-way ANOVA. Differences were considered statistically significant at $P < 0.05$. Data regarding probiotic features and functional properties of fermented juices were also analyzed by Principal Component Analysis (PCA), using Pearson correlation with the software XLSTAT (18.06).

3. Results and discussion

3.1. Screening for lactic acid bacteria

A previous study described the growth of naturally occurring lactic acid bacteria in *O. ficus-indica* fruits during storage, which was associated with the reduction or suppression of undesiderable microflora (Corbo et al., 2004). More recently, Di Cagno et al. (2016), isolated *Leuc. mesenteroides* strains from raw cactus pear fruits and were successfully used for the fermentation of cactus pear puree. Isolation of indigenous lactic acid bacteria from cactus pears is of particular interest to get improved quality and safety of fermented by-products from this fruit. This study aimed at isolating and selecting potential probiotic autochthonous lactic acid bacteria from *O. ficus-indica* fruits that growth in Argentina to obtain a fermented juice.

A total of 37 presumptive mesophilic and microaerophilic bacterial isolates were obtained from fresh *O. ficus-indica* fruits. These strains were randomly picked from the MRS agar plates, according to the apparent differences in the morphological appearance of the colonies (size, edge, color, and topography among others). Of these, 17 strains showed typical characteristics of lactic acid bacteria: Gram-positive, catalase-negative, non-motile, non-spore forming rods-cocci and cocci. All 17 isolates of lactic acid bacteria were able to grow between 15 and 37 °C and to acidify MRS broth. Eleven (11) isolates were able to grow at 42 °C, while only one (S-04) at 45 °C.

3.2. Cactus pear juice fermentation

The initial value of pH of the pasteurized juice was 5.4 and remained constant during the 48 h of incubation at 37 °C and during the 30 days of storage in refrigerator (4 °C). All 17 lactic acid bacteria were able to grow on pasteurized fruit juice without nutrient supplementation. All isolates, except S-02, were able to reduce the juice pH bellow 4.5 after 48 h of growth at 37 °C (Table 1). Four (4) of them (S-04, S-811, S-TF1 and S-TF2) could be considered fast acidifying strains that decrease the pH values between 3 and 4 in the first 6 h of growth. After 24 h of fermentation, the values of pH were lower than 3.3 in 11 of the studied strains. Extending the fermentation beyond 48 h did not result in an important decrease in the values of pH of fermented juices. Acid production and reduction in pH, which prevents product contamination, is associated with a good microbial growth (high viable counts) of lactic acid bacteria (Rathore, Salmerón, & Pandiella, 2012). In a recent study Panda et al. (2017) fermented cactus pear (*Opuntia* sp.) juice using a collection strain, *Lactobacillus fermentum* ATCC 9338. These authors could successfully preserve juice of cactus pears through lactic fermentation. However, at the end of fermentation the cells of *L. fermentum* became inactive (Panda et al., 2017). Furthermore, the decrease in pH reached with this strain (pH of 4.1 at 48 h) is much lower than that obtained with many of the autochthonous strains isolated in the present work.

During storage of fermented juices at 4 °C for 30 days, the values of pH remained almost constant (data not shown). At 30 d of storage, 8 isolates (S-04, S-22, S-24, S-811, S-TF1, S-TF2, S-TF3, S-TF7) survived at values of pH lower than 4.4 and down to 2.5, corroborating the adaptation of indigenous bacteria to their source of origin (Table 1). A previous study reported that vegetable

Table 1
Cactus fruit juice fermentation by autochthonous LAB.

LAB isolate	pH of the cactus fruit juice[A]				Survival in stored juice (4 °C)[B]	
	6 h	12 h	24 h	48 h	10 d	30 d
S-01	5.0 ± 0.2^{a}	4.5 ± 0.2^{b}	3.1 ± 0.1^{c}	3.0 ± 0.1^{c}	−	−
S-02	5.4 ± 0.1	5.4 ± 0.1	5.2 ± 0.1	5.2 ± 0.2	−	−
S-03	5.4 ± 0.1^{a}	4.1 ± 0.2^{b}	3.2 ± 0.2^{c}	3.1 ± 0.1^{c}	+	−
S-04	3.6 ± 0.1^{a}	3.5 ± 0.1^{a}	3.1 ± 0.1^{b}	3.0 ± 0.1^{b}	+	+
S-05	5.4 ± 0.0^{a}	4.5 ± 0.1^{b}	3.1 ± 0.2^{c}	2.6 ± 0.1^{d}	−	−
S-16	5.4 ± 0.0^{a}	5.3 ± 0.1^{a}	3.0 ± 0.1^{b}	2.6 ± 0.1^{c}	−	−
S-17	5.4 ± 0.0^{a}	5.1 ± 0.2^{a}	2.6 ± 0.2^{b}	2.5 ± 0.1^{b}	−	−
S-18	5.1 ± 0.1^{a}	4.4 ± 0.2^{b}	3.3 ± 0.2^{c}	3.1 ± 0.2^{c}	−	−
S-19	5.4 ± 0.1^{a}	4.6 ± 0.2^{b}	3.2 ± 0.2^{c}	3.0 ± 0.1^{c}	−	−
S-20	5.3 ± 0.1^{a}	4.5 ± 0.2^{b}	3.0 ± 0.2^{c}	2.6 ± 0.1^{c}	−	−
S-22	5.0 ± 0.1^{a}	4.4 ± 0.1^{b}	4.3 ± 0.1^{b}	4.3 ± 0.2^{b}	+	+
S-24	5.1 ± 0.2^{a}	4.6 ± 0.2^{b}	4.5 ± 0.2^{b}	4.0 ± 0.3^{b}	+	+
S-811	3.0 ± 0.1^{a}	2.9 ± 0.1^{ab}	2.7 ± 0.1^{bc}	2.5 ± 0.1^{c}	+	+
S-TF1	3.1 ± 0.2^{a}	2.6 ± 0.1^{b}	2.6 ± 0.2^{b}	2.5 ± 0.1^{b}	+	+
S-TF2	3.0 ± 0.2^{a}	2.9 ± 0.1^{a}	2.7 ± 0.1^{ab}	2.5 ± 0.1^{b}	+	+
S-TF3	5.1 ± 0.2^{a}	5.0 ± 0.2^{a}	4.9 ± 0.1^{a}	4.0 ± 0.2^{b}	+	+
S-TF7	5.2 ± 0.1^{a}	5.0 ± 0.1^{ab}	4.8 ± 0.1^{b}	4.4 ± 0.1^{c}	+	+

[A] Isolates were inoculated in pasteurized cactus fruit juice (64 °C; 30min) and cultured during 48 h at 37 °C. The pH of each juice was measured at 6, 12, 24 and 48 h. The initial pH of pasteurized juice was 5.4. Means within a raw with different letters differ significantly ($P < 0.05$).

[B] Fermented juices were stored in a refrigerator (4 °C) for 10 and 30 days.

extracts exhibit protective effect on the viability of *Lactobacillus* species under acidic condition (Charalampopoulos, Pandiella, & Webb, 2002).

3.3. In vitro probiotic studies

3.3.1. Resistance to gastric juice and bile salts

Ability to tolerate the conditions of the upper gastrointestinal tract (low pH of gastric juice and high concentrations of bile salts) is a major characteristic of a strain with potential probiotic activity, allowing survival and subsequent colonization of the gastrointestinal tract. Most strains were resistant to pH 3, but not to a pH 2 gastric juice. S-22 and S-TF7 strains were not affected after 2 h incubation at pH 3; while S-811, S-TF1 and S-TF2 strains showed decreases in the number of viable cells from 0.4 to 1.0 orders of log cfu/ml (Table 2). Only 3 of 17 strains (S-22, S-811 and S-TF2) survived at pH 2 when were maintained at this pH for 2 h, showing (S-811) less than 1 log orders reduction in cell count. Although many of the isolates were unable to tolerate gastric solution of pH 2, they could be still exploited as probiotics. Using improved food matrices or encapsulation techniques can be achieved an increased tolerance of bacteria to acidic conditions (Todorov et al., 2012; Vinderola et al., 2008). Even conventional food components can act buffering stomach acid and improving bacteria resistance (Guglielmotti, Briggiler Marcó, Golowczyc, Reinheimer, & Quiberoni, 2007). Many lactic acid bacteria strains, such as *Leuc. mesenteroides* NRRL B-1149 isolated from the intestine of snakehead fish, meat starter *L. curvatus* RM10 or the well-known *L. rhamnosus* GG were considered as probiotic microorganisms despite only tolerate acidic conditions from pH 3 (Allameh, Daud, Yusoff, Saad, & Ideris, 2012; Guglielmotti et al., 2007; Shukla, Iliev, & Goyal, 2014).

The effects of bile salts on the survival of isolates are shown in Table 2. All strains assayed showed resistance to bile salts and were able to grow in the presence of 0.3–1.0 g/100 ml bile salt, concentrations similar to those normally found in the human small intestine (Vinderola et al., 2008). In general, the effect of bile salts on lactic acid bacteria is variable, and growth inhibition at concentrations above 0.3 or 0.6 g/100 ml were reported in strains of *L. delbrueckii*, *L. paracasei*, *L. pentosus* and *L. rhamnosus* (Guglielmotti et al., 2007; Todorov et al., 2012). However, high levels of tolerance to bile salts were also described in other strains of lactic acid bacteria such as *L. plantarum*, *L. acidophilus*, *L. salivarius* and *L. curvatus*, which were able to resist between 1.0 and 3.0 g/100 ml bile salts (Shukla et al., 2014; Todorov et al., 2012). The resistance of these microorganisms to bile salts may be related to a general mechanism for adaptation to the stress which can be activated by various stress stimuli, including an acidic environment (Shukla et al., 2014).

3.3.2. Cell surface properties

Hydrophobic nature of the surface of microbes is related to its adhesion capacity to intestinal mucosa. In general, most of the isolates displayed low hydrophobicity (Table 2). Only five strains (S-02, S-22, S-24, S-TF3 and S-TF7) showed values above 40%. The hydrophobicity recorded for S-22 strain (64.3%) was similar to that observed in probiotic *L. acidophilus* M92 and commercial strain *L. rhamnosus* GG (both approximately 70%) and much higher than hydrophobicity values of probiotic strains *L. plantarum* DGK-17, *L. casei* SB93 and *L. mucosae* (12.6, 25.0 and 25.9%, respectively), all measured using the same solvent (*p*-xylene) (Caggia, De Angelis, Pitino, Pino, & Randazzo, 2015; Das, Khowala, & Biswas, 2016; Khan & Kang, 2016; Kos et al., 2003).

Regarding to autoaggregation ability, the values obtained were consistent with those of hydrophobicity. Most hydrophobic strains (S-02, S-22, S-24, S-TF3 and S-TF7) displayed the higher values of autoaggregation (between 25.1 and 62.5%). Only one isolate (S-TF7) exhibited autoaggregation and hydrophobicity values higher than

Table 2
In vitro probiotic properties of isolated LAB.

Isolate	Resistance to Gastric solution[a]		Inhibition of growth in presence of bile (%; w/v)[b]		Hydrophobicity (%)	Autoaggregation (%)	FEA	Inhibition of *Bacillus* sp.[c]	
	pH 2	pH 3	1.0	2.0				Well-diffusion agar[d]	"*In* situ" inhibition[e]
S-01	−	+	−	2	0.9 ± 0.1	5.3 ± 0.4	−	0	-
S-02	−	−	−	4	48.0 ± 2.6	45.2 ± 4.1	−	0	ND
S-03	−	+	−	1	3.0 ± 0.2	10.6 ± 0.6	−	0	ND
S-04	−	+	−	−	5.7 ± 0.3	5.8 ± 0.3	−	0	ND
S-05	−	+	−	1	4.8 ± 0.3	6.5 ± 0.4	−	0	ND
S-16	−	+	−	4	0	13.3 ± 0.9	−	0	ND
S-17	−	+	−	2	0	3.6 ± 0.3	−	0	ND
S-18	−	+	−	2	14.2 ± 0.8	9.0 ± 0.8	−	0	ND
S-19	−	+	−	4	10.2 ± 0.5	1.5 ± 0.1	−	0	ND
S-20	−	−	−	4	0	6.3 ± 0.5	−	0	ND
S-22	+	++	−	2	64.3 ± 3.7	25.1 ± 2.1	−	3.0	-
S-24	−	+	−	1	41.1 ± 2.8	39.3 ± 4.1	−	0	ND
S-811	++	++	−	−	10.2 ± 0.5	7.4 ± 0.5	+	4.0	+
S-TF1	−	++	−	−	4.9 ± 0.3	8.7 ± 0.4	+	2.0	+
S-TF2	+	++	−	−	3.5 ± 0.3	9.9 ± 0.3	+	2.0	+
S-TF3	−	+	−	−	46.3 ± 4.0	61.3 ± 2.0	−	0	ND
S-TF7	−	++	−	−	53.5 ± 5.2	62.5 ± 3.1	−	2.0	-

FEA: Feruloyl esterase activity.

[a] Each isolate was incubated in simulated gastric juice (pH 2 or 3) during 2 h at 37 °C; (−), no survival; (+), decrease in viable cell counts (Δlog cfu/ml) after exposure to gastric solution > 1.0; (++), Δlog cfu/ml ≤ 1.0.

[b] Inhibition halo diameter (mm); (−), absence of inhibition; well diameter (5 mm).

[c] Strain of *Bacillus* sp. isolated from decaying cactus pear juice.

[d] Inhibition halo diameter (mm) of fermented juices against *Bacillus* sp.; (0), absence of inhibition; well diameter (5 mm).

[e] *Bacillus* sp. was inoculated in fermented juices that showed inhibitory effect on it in the well-diffusion agar assay. **ND,** not determined. **(−),** no inhibition of *Bacillus* sp.; **(+),** inhibition of *Bacillus* sp.

50%. The autoaggregation percentage for S-TF3 and S-TF7 isolates (61.3 and 62.5%, respectively) were close to that measured in *L. casei* SB71 (59.8%) and commercial probiotic *L. rhamnosus* GG (approximately 70%) (Das et al., 2016). Both hydrophobicity and autoaggregation contributes to the adhesion of bacteria to intestinal mucosa, but they are not a requirement for a strong adherence capacity (Todorov et al., 2012). Furthermore, evidence indicated that hydrophobicity and autoaggregation are strain dependent properties, varying greatly among even the same bacteria species (Caggia et al., 2015; Das et al., 2016). Good hydrophobicity and autoaggregation values not necessarily mean an *in vivo* adhesion, where host factors are involved, such as defense mechanisms, resident microbiota, and peristaltic flow that can modify the bacterial adhesion (Caggia et al., 2015).

3.3.3. Feruloyl esterase activity

Feruloyl esterases are carboxyl esterases that release ferulic acid from different sources. Of the 17 stains screened on MRS agar plates containing ethyl ferulate only 3 isolates, S-811, S-TF1 and S-TF2, showed feruloyl esterase activity (Table 2) Beneficial effects of administration of feruloyl esterase-producing LAB was reported (Abeijon Mukdsi et al., 2013; Russo et al., 2016). These authors described increases of feruloyl esterase activity in intestinal mucosa of mice administered with feruloyl esterase-producing *L. fermentum* CRL1446. This increment in enzyme activity was accompanied of an increase in plasma of the antioxidant enzyme glutathione reductase and decreases in lipid peroxidation.

3.3.4. Antimicrobial activity of fermented cactus pear juices

The antibacterial activity of the fermented juices was assessed on a strain of *Bacillus* sp. isolated from decaying cactus pear juice. Only juices fermented with S-22, S-811, S-TF1, S-TF2 and S-TF7 isolates showed some antibacterial effect against the juice contaminant *Bacillus* sp. (Table 2). The inhibition halo diameters obtained with these fermented juices ranged from 2 to 4 mm. When *Bacillus* sp. was inoculated in juices fermented and maintained during 1 h at 37 °C, a complete inhibition of this bacterium was observed in juices fermented with S-811, S-TF1 and S-TF2 (Table 2). In general, antimicrobial activity exerted by LAB is due to the presence of a mixture of protein and acidic-like substances (lactic acid mainly), these last ones acting directly or through the fall in pH of foods (Guglielmotti et al., 2007; Vinderola et al., 2008). The isolates S-05, S-16, S-17 and S-20, in spite of acidifying cactus fruit juice to pH < 3 (similar to S-811, S-TF1 and S-TF2 isolates) they did not manifest antibacterial activity against *Bacillus* sp.

3.4. Phenolics and betalains in fermented cactus pear juices

A protocol to carrying out cactus pear juice fermentation was set up. Since the pH of cactus fruit juice changes very little after the hour 24 of culture, lactic acid fermentation was stopped upon completion of 24 h. Control and fermented juices were stored in refrigerator (4 °C) for 30 days Total phenolic and betalains contents were analyzed at 0, 10 and 30 days of storage. The green cactus pear juices fermented with the autochthonous isolates and not fermented showed a low betalains content (about 7 mg/l indicaxanthin and about 8 mg/l betanin) that did not significantly ($P > 0.05$) vary during storage. These results are in agreement with those reported by Jiménez-Aguilar et al., (2015), who did not detect betalains pigments in green varieties of cactus pears. This is in contrast to the high values measured (up 50 times higher) in the purple, red and orange varieties (Cassano et al., 2007; Jiménez-Aguilar et al., 2015). The total phenolic compounds content in fermented juices at day 0 (between 682 and 808 mg GAE/l; equivalent to 5.18 and 6.14 mg GAE/g DW, respectively) and after 10 and 30 days of storage at 4 °C was not significantly ($P > 0.05$) different with respect to unfermented control juice of the same storage time (714 mg GAE/l at day 0; equivalent to 5.43 mg GAE/g DW) (Fig. 1). These results are in agreement with the findings of Di Cagno et al. (2016), who reported that lactic fermentation and storage did not affect phenolic levels. Both, the LAB isolate used as starter and the storage time affected significantly (F-value = 2.79 and 36.35, respectively; $P < 0.001$) the phenolics content in the juices, but no interaction between these factors was recorded ($P > 0.05$). These concentrations of phenolic compounds were remarkable higher than those observed in pulp of a green variety of *O. ficus-indica* fruit (0.6 mg GAE/g DW) collected in other region of Argentina and similar to values reported for the juice of a Mexican green variety (4.71 mg GAE/g DW) (Coria Cayupán et al., 2011; Jiménez-Aguilar et al., 2015). It is known that the presence of phenolic compounds in fruits, vegetables and foods can inhibit the oxidative processes that promote their spoilage and hence positively contributing to their conservation (Abdel-Hameed, Nagaty, Salman, & Bazaid, 2014).

3.5. Antioxidant capacity of fermented cactus pear juices

Previous studies showed that cactus fruits have free radical scavenging activities similar to that observed in citrus and other seasonal fruits (Coria Cayupán et al., 2011). The free radical scavenger potential of juices was assay by the ABTS test. All juices were scavengers with SC_{50} values between 3.3 and 5.7 mg GAE/l (Fig. 2). In general, fermented juices showed similar antioxidant activity than control juice, except juices fermented with strains S-18, S-22 and S-24, that were significantly more active than control juice. The analysis of the baseline values of antioxidant activity in each juice showed that the effect of storage was not the same in all the juices. Juices fermented with isolates S-03, S-811, S-TF1, S-TF2, S-TF3 and S-TF7 displayed a significant ($P < 0.05$) increment in their antioxidant capacities after 10 or 30 days of storage at 4 °C. This increase in antioxidant activity is possibly related to variations in phenolics profiles (Butera et al., 2002; Di Cagno et al., 2016). Furthermore, some of these juices (S-TF1, S-TF2 and S-TF7), showed higher ($P < 0.05$) antioxidant activity than unfermented juice after 30 days of storage at 4 °C (Fig. 2). Similar effect was observed by Di Cagno et al. (2016) in cactus pear puree fermented with *Leuc. mesenteroides* strains. The storage did not vary significantly ($P > 0.05$) the antioxidant activity of juices fermented with isolates S-22 and S-811 in comparison with control unfermented juice stored under the same conditions. These results suggest that phenolic compounds could contribute to the antioxidant capacity of fermented juices. Some of these compounds may be also produced by some of the isolated microorganisms. Di Cagno et al. (2016) hypothesized that the positive contribution to the antioxidant activity in fermented cactus pear puree is linked to variations of the phenolic compounds profiles. Contrary to what was observed in most of the cactus pear juices fermented with autochthonous strains, cactus pear juice fermentation using allochtonous *L. fermentum* ATCC 9338, resulted in a decrease of about 30% in antioxidant activity of juice (Panda et al., 2017). Additionally, analysis performed by the two-way ANOVA showed a significant effect due to the type of LAB isolate used as starter (F-value = 37.35; $P < 0.001$), and the storage time (F-value = 22.46; $P < 0.001$) on antioxidant capacity of fermented juices. Also, there was significant interaction (F-value = 36.36; $P < 0.001$) between these factors (LAB starter and storage time) and their effect on antioxidant capacity of juices.

3.6. Identification of selected lactic acid bacteria

Fig. 3 shows the principal component analysis (PCA) with focus

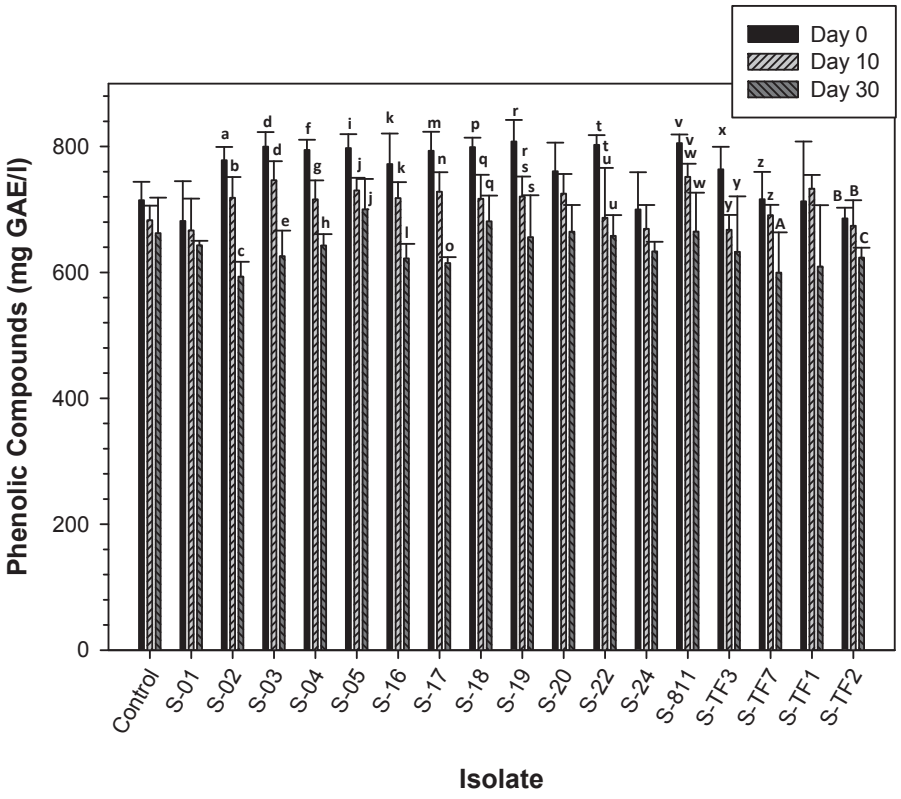

Fig. 1. Total phenolic compounds content of fermented cactus pear juices (24 h; 37 °C) at 0, 10 and 30 days of storage in refrigerator (4 °C). Control: unfermented pasteurized juice. Results were expressed as mg of gallic acid equivalents per l of juice (mg GAE/l). One-way ANOVA with Tukey Post Hoc test was performed to compare differences across juices stored for 0, 10 and 30 days with the control juice stored for the same period, and between baselines in each juice. Different letter denotes that values are significantly different at a significance level of $P < 0.05$ between baselines in each juice. No significant differences ($P > 0.05$) with the respective control juice of the same age were found in any fermented juice.

on groupings of isolates with respect to probiotic features of the microorganisms and their effect on functional properties of fermented cactus pear juice. The first and second factors accounted for 65.42% of the total variance, and considering also the third component about 76% of the total variance can be explained (third component is not presented graphically). The first principal component discriminated better the isolates according to their beneficial probiotic properties, and was more influenced by *in situ* antimicrobial activity and feruloyl esterase activity. Isolates S-22, S-811, S-TF1, S-TF2 and S-TF7 were grouped on the same zone of the plane regarding the first component due they shared positive attributes as survival on stored juice, better tolerance to gastrointestinal conditions, feruloyl esterase activity and antimicrobial activity. Second and third components separate the isolates with regard to their cell surface properties and phenolics content, respectively. Isolates S-22, S-TF3 and S-TF-7 are among those with higher values of hydrophobicity and autoaggregation, while cactus pear juice fermented with LAB S-811 had the higher phenolic compounds concentration. Based on the probiotic potential of the isolated LAB and the effect thereof on the functional properties of the cactus pear juice, four (4) isolates were selected as candidates for the production of a fermented beverage from fruits of *O. ficus-indica*. In a recent work, Di Cagno et al. (2016) suggested that cactus pear fruits represent a very selective environment, since only strains of *Leuc. mesenteroides* species could be isolated from these fruits. The LAB selected in this study were identified by partial sequencing of the 16S rRNA as *Lactobacillus plantarum* S-811, *Lactobacillus plantarum* S-TF2, *Fructobacillus fructosus* S-22 and *Fructobacillus fructosus* S-TF7 strains (Table 3).

Previous studies have shown that fruit juices and other vegetables foods fermented with autochthonous strains have better performance with respect to unstarted or fermented with allocthonous starter. Particularly, vegetables fermented with autochthonous strains of *L. plantarum* have better viscosity, color, flavor and health-promoting profile compared to those fermented with allocthonous starters (Di Cagno et al., 2013). *L. plantarum* is between the main species isolated from raw and fermented vegetables (Di Cagno et al., 2013). Usually, in spontaneously fermented vegetables *L. mesenteroides* started the first stage of fermentation but it is inhibited by the increasing concentration of lactic acid, and then a combination of *L. plantarum* and other acid-tolerant *Lactobacillus* species continue fermentation (Di Cagno et al., 2013). Indeed, several potential probiotic strains of *L. plantarum* were variously isolated from fruits and vegetables such as cabbage, carrots, lettuce, peppers, cucumbers, zucchinis, eggplants, redbeet, fennel, and capers, as well as of olives, tomatoes, pineapple, kiwi, plums, papayas and cherries (Peres, Peres, Hernandez-Mendoza, & Malcata, 2012; Hurtado, Reguant, Bordons & Rozès, 2012; Di Cagno et al., 2013). Many of these strains showed resistance to gastroduodenal stress, and the capacity of adhesion to the gut epithelium, the ability to hydrolyse nutritional constituents, which cannot be metabolized by the host (e.g., fructooligosaccharides), and to synthesize antimicrobial compounds active towards pathogenic Gram positive bacteria (*B. cereus, L. monocytogenes* and *S. aureus*), Gram negative bacteria (*E. coli*, *Salmonella* spp. and *Salmonella enterica*) and yeasts (Peres et al., 2012). Many of these strains were efficiently used, alone or in combination with other *Lactobacillus* species, for the fermentation and conservation of fruit juices and

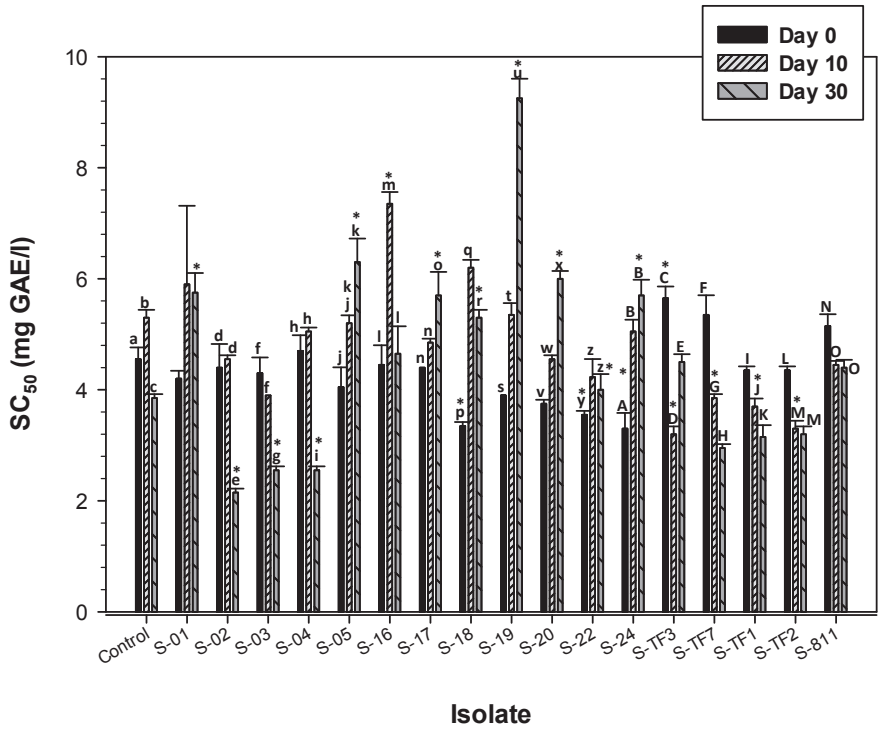

Fig. 2. Total antioxidant activity (scavenging activity of ABTS radical cation) of fermented cactus fruit juices (24 h; 37 °C) at 0, 10 and 30 of storage in refrigerator (4 °C). Control: unfermented pasteurized juice. Results were expressed as SC_{50} (mg of GAE/l). One-way ANOVA with Tukey Post Hoc test was performed to compare differences across juices stored for 0, 10 and 30 days with the control juice stored for the same period, and between baselines in each juice. Different letter denotes that values are significantly different at a significance level of $P < 0.05$ between baselines in each juice. *Denotes statistical significance at $P < 0.05$ with the respective control juice of the same age.

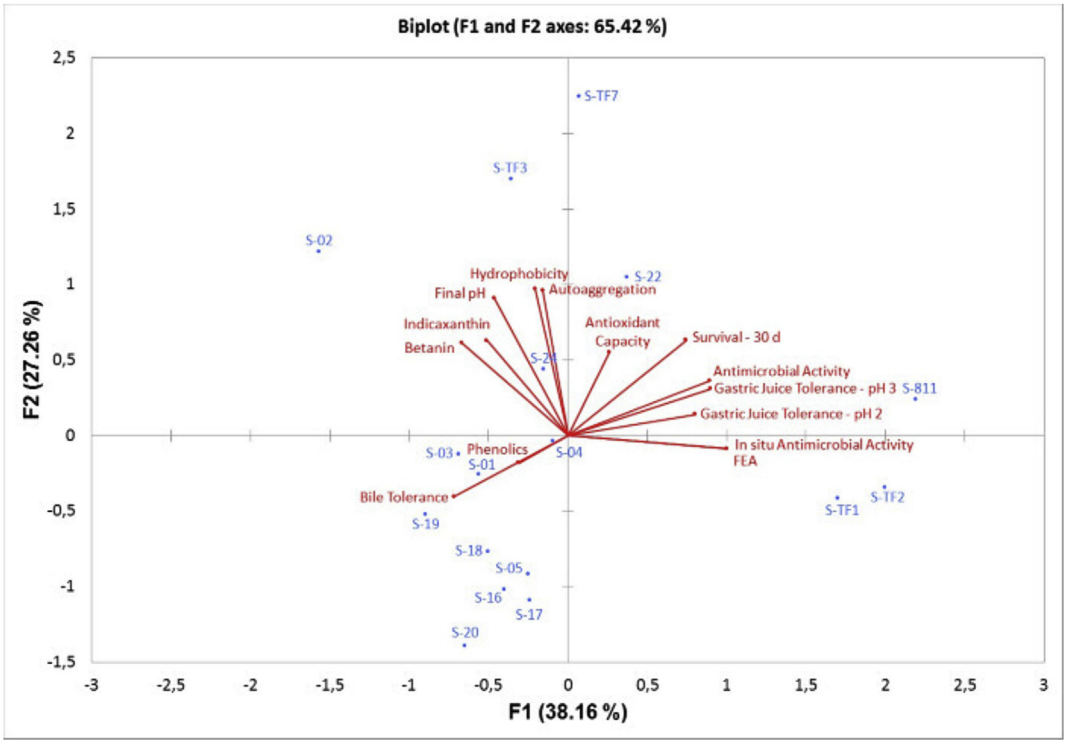

Fig. 3. Principal component analysis (PCA) biplot, based on data regarding to probiotic features of the isolates and the functional properties of fermented cactus pear juices. PCA explains 65.42% of the total variation distributed in F1 38.16% and F2 27.26%.

Table 3
16S rRNA gene sequencing of selected strains isolated from *O. ficus-indica* and analysis by BLAST and RDP SeqMatch (S_ab score).

Strain	16S rRNA gene sequencing data (closest relative specie)	Identity (%) BLAST	S_ab score	GeneBank Access number
S-22	*Fructobacillus fructosus*	99.55	1	KJ794639
S-811	*Lactobacillus plantarum*	99.78	1	KJ787685
S-TF2	*Lactobacillus plantarum*	99.57	1	KJ794638
S-TF7	*Fructobacillus fructosus*	99.55	1	KJ794640

vegetables and the elaboration of probiotic beverages (Peres et al., 2012; Di Cagno et al., 2013). Pomegranate juices fermented with *L. plantarum* showed improved functional and sensory properties, affecting positively immunomodulatory capacity, phenolic compounds contents and antioxidant activity of the beverage (Filannino et al., 2013).

The genus *Fructobacillus* belongs to the group of fructophilic LAB which prefer to use fructose as carbon source rather than glucose. Until 2008 this genus was considered as a subgroup of *Leuconostoc fructosum*, when it was reclassified as *Fructobacillus* genus (Endo & Okada, 2008). These bacteria usually inhabit in fructose-rich niches, mainly fruits, flowers and also the intestine of some insects (He, Chen, Zhang, & Wei, 2011). *Fructobacillus* spp. grows well on D-fructose, but poorly on D-glucose without external electron acceptors such as oxygen or pyruvate (Endo, Futagawa-Endo, Sakamoto, Kitahara, & Dicks, 2010). These are heterofermentative bacteria that produce lactic acid and acetic acid from D-glucose or D-fructose (Endo, Futagawa-Endo, & Dick, 2009). Isolation of bacteria belonging to the genus *Fructobacillus* is scarce, as evident from the few scientific papers published on *Fructobacillus* spp. (Endo et al., 2009). Recently, *Fructobacillus* species were isolated from fermented cocoa, fermented Blaufränkisch grapes during the vinification process and from tempoyak, a traditional fermented condiment made from the pulp of the durian fruit in Malaysia and Indonesia (Chuah et al., 2016; Godálová et al., 2016; Pereira, Soccol, & Soccol, 2016). However, practically there are no studies about probiotic properties of these bacteria, nor a history of their use in the preparation of fermented foods. Most investigations conducted to these fructophilic LAB was directed to the study of systematic and genetic diversity (Endo & Okada, 2008; Endo et al., 2009; Chelo, Zé-Zé, & Tenreiro, 2010.). Up to now, only two studies explores some microbiological, probiotic or technological characteristics of *Fructobacillus fructosus* species (Kelanne, 2012; Prückler et al., 2015). Two strains of *F. fructosus* isolated from flowers showed good tolerance to heat shock (70 °C) and moderate tolerance to gastric juice and bile salts. Furthermore, Prückler et al. (2015) reported the technological potential for wheat bran fermentation of *F. fructosus* FF14-1 strain. To our knowledge, *F. fructosus* S-22 and S-TF7 strains are the first *Fructobacillus* spp. isolated from fruits of *O. ficus-indica*, and the first isolated following criteria for selecting LAB with probiotic potential.

4. Conclusions

Cactus pear represents a pleasant flavor and complete food that provides nutrients and functional compounds which give health-promoting properties. Nevertheless, the fruit is susceptible of rapid microbial spoilage limiting its commercial exploitation. The fermentation of cactus fruit juice by autochthonous LAB emerges as a viable alternative to exploit this abundant natural resource in Northwestern Argentina. In this work, autochthonous *L. plantarum* and *F. fructosus* strains were selected as potential probiotic bacteria starters for the fermentation of cactus pear juice and develop new functional beverages. These strains are able to acidify juice and inhibit a contaminating bacterium, guaranteeing juice preservation and the conservation of its health-promoting features. Currently, an evaluation of beneficial health effects of fermented juices in obesity animal models is being carried out, regarding their effects on the improvement of altered metabolic parameters and weight loss. Furthermore, extensive studies should be performed, especially in regard to the safe use of the selected strains and the sensory properties of the fermented juices.

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