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Nonstarter Lactobacilli Isolated from Soft and Semihard Argentinean Cheeses: Genetic Characterization and Resistance to Biological Barriers

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ABSTRACT

Nonstarter lactic acid bacteria isolated from Argentinean cheeses were identified and characterized by focusing on their resistance to biological barriers, along with other physiological features of potential interest, in the search for future probiotic organisms. Lactobacilli were enumerated and isolated from semihard and soft cheeses made with multistrain *Streptococcus thermophilus* starters. Lactobacilli counts in 1-week-old cheeses were between 10^5 and 10^7 CFU/g and then reached 10^7 CFU/g in all 1-month samples, while streptococci were always above 10^9 CFU/g. A total number of 22 lactobacilli isolates were retained, identified, and characterized by in vitro tests. Species identity was determined by carbohydrate metabolism and species-specific PCR assays. Genetic diversity was explored by random amplified polymorphic DNA (RAPD) PCR analysis. The *Lactobacillus* strains were assigned to the species *L. casei, L. plantarum, L. rhamnosus, L. curvatus, L. fermentum,* and *L. perolens.* All the strains studied tolerated 25 ppm of lysozyme, and most of them showed resistance to 0.3% bile. After incubation in gastric solution (pH 2.0), counts decreased by several log units, ranging from 3.2 to 7.0. The strains were able to grow in the presence of bile salts, but only three isolates were capable of deconjugation. The nonstarter lactobacilli that were assayed fermented the prebiotic substrates (especially lactulose and inulin). Some strains showed high cell hydrophobicity and β-galactosidase activity, as well as inhibitory activity against pathogenic bacteria. It was concluded that most of the lactobacilli isolated in this study demonstrated resistance to biological barriers and physiological characteristics compatible with probiotic properties, which make them suitable for further research in in vivo studies aimed at identifying new probiotic organisms.

Nonstarter lactic acid bacteria are adventitious lactic acid bacteria that contaminate cheeses. In contrast to starter lactic acid bacteria, which are inoculated into cheese milk at high cell concentrations and decline during the ripening period, nonstarter lactic acid bacteria initial counts are very low (10^2 to 10^4 CFU/ml in good-quality commercial raw milk and 10 to 10^4 CFU/g in 1-day-old Cheddar cheese manufactured with pasteurized milk), but they increase (up to 10^7 to 10^8 CFU/g) during the first few weeks of ripening *(15).* Nonstarter lactic acid bacteria are mainly composed of lactobacilli in cheeses manufactured with pasteurized milk, but they may also include pediococci and enterococci *(8, 32).* The origin of nonstarter lactic acid bacteria in cheese is probably raw milk *(44).* Alternatively, lactobacilli may be part of the resident flora in the dairy production plant *(34)* and may contaminate cheese milk after pasteurization *(25).*

Nonstarter lactobacilli have been indicated as the only uncontrolled factor in today's industrial cheese making processes and are consequently the main source of quality inconsistencies and defects in cheese products *(10).* However, other authors believe that nonstarter lactobacilli can contribute in a positive way to the diversity and enhancement of cheese flavor *(21, 22).* Because no effective strategy for restraining nonstarter lactic acid bacteria development in cheese is yet known, the alternative of indirectly controlling secondary microflora in cheese by means of adjunct culture addition has been suggested. For that purpose, an important amount of research has been committed to isolating and characterizing strains of nonstarter lactic acid bacteria from good-quality cheeses. Most available strains have been obtained from Cheddar cheese, although recent studies have included lactobacilli isolated from ewe's milk and goat's milk cheeses *(11, 37).* The presence and biochemical activities of nonstarter lactic acid bacteria have been related to cheese flavor through their impact on proteolysis and amino acid catabolism. Cheeses with higher counts of nonstarter lactic acid bacteria have a higher free amino acid content and enhanced flavor *(22, 23).* The key enzymes involved in amino acid catabolism have been found in several nonstarter *Lactobacillus* strains, suggesting that nonstarter lactic acid bacteria contribute to cheese flavor *(43).*

A second aspect of the presence of lactobacilli in cheese, both as adventitious flora and in adjunct cultures, has been less well explored: their potential role as probiotic organisms. Even though definitions of probiotic bacteria include intestinal origin as a requisite for this status, many

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nonstarter lactobacilli belong to the species *Lactobacillus casei/paracasei/plantarum*, which also host probiotic strains. For that reason, the search for resistance to biological barriers and physiological characteristics compatible with probiotic properties among lactobacilli isolated from cheese may eventually lead to the finding of new probiotic strains for functional dairy foods *(3, 24)*.

The objective of the present study was to quantify and isolate nonstarter lactic acid bacteria from good-quality Argentinean cheeses and then to identify and characterize the isolated *Lactobacillus* strains by means of genetic techniques and in vitro studies that focus on the search for potential future probiotic organisms.

MATERIALS AND METHODS

Cheese samples. Nonstarter lactic acid bacteria were isolated from five cheeses produced in three industrial dairy plants located in the Santa Fe (Argentina) area. Even though nonstarter bacteria have been mainly studied in long-ripened cheese varieties, for this study, we selected semihard and soft cheeses. This choice was made to facilitate the isolation of nonstarter lactobacilli, as Argentinean hard cheeses are produced with primary starters composed of thermophilic lactobacilli. Among the selected samples, some were discarded as a source of lactobacilli, as we found that the starter contained *Lactobacillus delbrueckii* subsp. *bulgaricus*, which was then confirmed by the cheese makers. The cheese varieties Cremoso Argentino (one sample), Holanda Argentino (one sample), Tybo Argentino (two samples, A and B [Tybo A and B]), and Pategrás Argentino (one sample), manufactured with a direct-to-vat primary starter composed of *Streptococcus thermophilus* strains only, were then chosen for this study. We also studied a semihard cheese (obtained from a farm) made with raw milk and a commercial starter of *S. thermophilus*. Whole pieces of young or ripened cheeses were supplied by the cheese makers and stored in our ripening chamber at 12°C and 80% relative humidity. The youngest cheeses—Pategrás cheese, Cremoso cheese, and the raw milk semihard cheese—were 1 week old. They were kept in the chamber for 3 months and sampled during ripening. Ripened cheeses were between 3 and 5 months old and were sampled twice, first when they arrived at our laboratory and then a month later.

Microbial counts. Five to ten ~10-g cylinders were taken from the cheeses with a sterile sampler and ground aseptically. Then, a 20-g sample was homogenized for 3 min in a stomacher lab blender (PBI International, Milan, Italy) with sterile sodium citrate solution (2% wt/vol). From cheese homogenates, decimal dilutions were made in 0.1% (wt/vol) sterile peptone water.

For nonstarter lactic acid bacteria counts, a set of different culture media were evaluated: deMan Rogosa Sharpe (MRS) agar (Biokar, Beauvais, France), bile-MRS agar *(48)*, acid-MRS agar (pH 5.5) *(18)*, and Elliker agar NaCl (6.5% NaCl, wt/vol; Biokar) for lactobacilli. Surface platings were made, and the plates were incubated for 48 h at 34°C. Lactic acid starter bacteria (*S. thermophilus*) were enumerated on skim milk agar (48 h at 37°C) *(35)*. Coliform bacteria and mold and yeast counts were performed on violet red bile agar (24 h at 30°C) and yeast extract glucose chloramphenicol agar (Britania, Buenos Aires, Argentina) (5 days at (×1,000), motility, catalase activity, gas production in M containing Durham tubes, and cell agglutination in bro positive, catalase-negative, nonmotile rods were retain nonstarter lactic acid bacteria lactobacilli and were sto at −80°C in MRS broth supplemented with 15% (vol/ crol.

Lactobacilli isolates were identified according to *Manual of Systematic Bacteriology (17)*. Sugar metabo terns were evaluated with API 50 CHL identification System, bioMérieux, Montalieu-Vercieu, France) accord manufacturer's instructions.

Genetic characterization. Total cDNA was extra the overnight MRS broth culture (approximately 10^7 CF were pelleted by centrifugation and then washed twice w (10 mM Tris-HCl and 0.1 mM EDTA [pH 8.0]) and r The DNA was extracted by a Chelex-based method acc the procedure for gram-positive (and acid-fast) bacteria in the MicroSeq protocol (Applera Italia, Monza, Italy)

For the *L. casei, Lactobacillus rhamnosus*, and *L. p* isolates, species identity was confirmed by species-spe assays *(4, 13)*. *Lactobacillus* strains that were unidenti species-specific PCR (*L. curvatus, L. fermentum*, and *L.* isolates) were identified by sequencing the hypervariab (first 500 bp) in the 5′ region of the 16S rRNA gene. analysis, the MicroSeq 500 16S rDNA Bacterial Sequer Identification kit system was used (Applera Italia). Seq sessment and data analysis were performed as describe setti and Giraffa *(36)*.

Random amplified polymorphic DNA (RAPD) PCR to explore lactobacilli diversity. cDNA from different st used for PCR fingerprinting, with the M13 minisatellite quence as a primer with the sequence 5′-GAGGGTGGC 3′ *(36)*. PCR profiles were visualized after overnight electr in agarose gels (Celbio spa, Milan, Italy) after staining with bromide; a DNA ladder (Invitrogen srl, Milan, Italy) was DNA molecular-weight marker. The images of the gels v tured, exported, and processed as previously reported *(36,*

Characterization of strains. Strain tolerance to s gastric juice was determined with a solution of pepsin (0 vol; Tuteur SACIFIA, Buenos Aires, Argentina) and NaC wt/vol) adjusted to pH 2. Overnight cultures of the stra centrifuged, washed, and resuspended in phosphate buffer K_2HPO_4 [pH 6.5]) and then harvested and resuspend phosphate buffer (pH 6.5) or (ii) simulated gastric juice. pensions were maintained at 37°C for 3 h. Total viable c MRS agar of the solutions before and after this incubati determined, and the results were expressed as the differe tween each pair of counts (log CFU per milliliter) *(7)*.

Bile resistance was studied by inoculating strains broth, which contained 0.3, 0.5, or 1% bile (Sigma, St Mo.). Cultures were incubated at 37°C, and after 24 h growth, the A_{560} was measured and compared to a control the addition of bile. The results were expressed as the pe of growth in the presence of bile compared to the contr Lysozyme resistance was assessed similarly, by inoculating in MRS broth with 25, 50, and 100 ppm of lysozyme and comparing lactobacilli growth to a control without lys

The ability of the isolates to metabolize several c

Sigma). Modified MRS broth consisted of basic MRS broth (without glucose) supplemented with 2% (wt/vol) of each prebiotic. Results were expressed as stated above, taking into account the growth (A_{560}) of the strains in the broth with the addition of a prebiotic and compared with a control incubated in standard MRS (with glucose and without prebiotics).

The ability of lactobacilli strains to facilitate bile salt deconjugation was examined by streaking overnight cultures of each isolate on petri plates that had been prepared by adding 0.5% (wt/vol) of each bile sodium salt to MRS agar. Tested sodium salts were those of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), and glycodeoxycholic acid (GDC), all from Sigma. After anaerobic incubation (GasPack System, Oxoid, Basingstoke, Hampshire, UK) at 37°C for 3 days, the presence of an opaque halo of precipitated bile acid around colonies was considered an indication of bile salt deconjugation *(42)*.

To assess hydrophobicity, suspensions were prepared with cells harvested from overnight stationary-phase cultures and then mixed with *n*-hexadecane (5:1, respectively) and vortexed *(31)*. After phase separation, the A_{560} value was measured in the aqueous layer. The cell surface hydrophobicity (%H) was calculated as follows:

$$\%H = [(A_0 - A)/A_0] \times 100$$

where A_0 and A are the values of absorbance before and after extraction with the organic solvent, respectively *(49)*.

The β-galactosidase activity of whole cells was determined according to the method of Miller *(26)*, modified as follows. Overnight stationary-phase cultures were harvested by centrifugation, washed twice, suspended in phosphate buffer (pH 6.5), and inoculated (1%) into lactose-MRS broth. After incubation at 37°C for 24 h, cells were harvested, washed twice, and resuspended in the same buffer to obtain suspensions with an $A_{560} = 1$. Aliquots of the suspensions were permeabilized with toluene-acetone solution and assayed for β-galactosidase activity with *o*-nitro-β-D-galactopyranoside (Sigma) as the reaction substrate. After incubation at 37°C for 15 min, the reaction was stopped with 1 M Na_2CO_3 solution. Absorbance at both 420 and 560 nm was determined, and β-galactosidase activity was calculated (in Miller units) as follows:

β-galactosidase

$$= 1{,}000 \times [(A_{420} - 1.75 \times A_{560}{}^a)/(15 \text{ min} \times 1 \text{ ml} \times A_{560}{}^b)]$$

where $A_{560}{}^a$ is the cell density before the assay, and $A_{560}{}^b$ is the cell density of the reaction mixture.

Finally, the well-diffusion agar assay was used to test antibacterial activity in the isolates. Cell-free extracts were obtained by the centrifugation of overnight cultures and then the filtration of these cultures through a 0.45-μm-pore-size filter to sterilize the supernatant (Millipore, Biopore SR1., Buenos Aires, Argentina). For the preparation of plates containing pathogens, nutrient agar (for *Salmonella, Staphylococcus aureus,* or *Escherichia coli*) or brain heart infusion agar (for *Listeria monocytogenes*) was melted and tempered at 45°C and then vigorously mixed with an overnight culture of a pathogen ($A_{560} = 0.8$) and poured onto a petri dish. Pathogen strains used in the assays were as follows: *Salmonella* Enteritidis OMS-Ca (isolated from mayonnaise, INLAIN collection), *S. aureus* 76 (isolated from raw milk, INLAIN collection), *E. coli* V157 (kindly supplied by the Istituto di Microbiologia—Università Cattolica del Sacro Cuore Sede di Piacenza, Italia), and *L. monocytogenes* ATCC 15313. Wells with a diameter of 6 mm were filled with 50 μl of cell-free extract and incubated overnight at 37°C, and the diameters of the inhibition zones were recorded *(47)*.

TABLE 1. *Starter and nonstarter lactic acid bacteria counts in cheeses*

Cheese[a]	Ripening time (days)	Colony counts (log CFU/g	
		Streptococci	Lactoba
Pategrás	10	9.34 ± 0.14	7.71 ±
	70	9.00 ± 0.12	7.85 ±
Tybo A	10	9.41 ± 0.06	5.90 ±
	70	8.57 ± 0.06	6.60 ±
Tybo B	10	9.04 ± 0.05	7.04 ±
	70	8.86 ± 0.11	7.49 ±
Cremoso	10	9.00 ± 0.16	7.23 ±
	70	8.27 ± 0.16	8.27 ±
Semihard raw milk cheese	10	8.99 ± 0.13	8.04 ±
	70	10.53 ± 0.08	9.41 ±

[a] Cheeses made with pasteurized milk were Pategrás, Tybo A, Tybo B, and Cremoso. Tybo B and Cremoso were from the same plant, Pategrás from the second plant, and Tybo A from the plant.

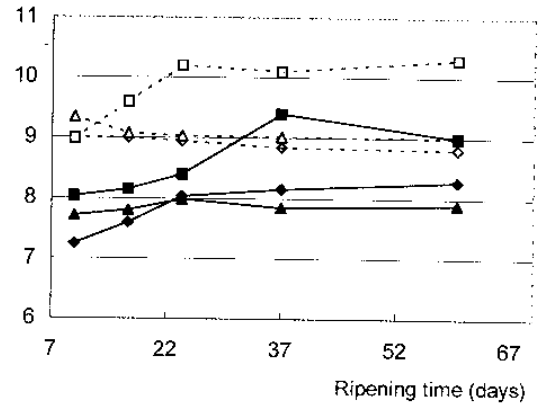
[b] Values are means ± standard deviations of three determina

RESULTS

Lactobacilli counts and identification. Plate co obtained on MRS agar were significantly higher than t observed on acid-MRS agar, bile-MRS agar, and El agar NaCl. Consequently, nonstarter lactic acid bac counts were recorded from MRS agar plates. Starter also able to grow on this culture medium, but colonie *S. thermophilus* showed a characteristic morphology was completely different from lactobacilli, and there they did not interfere in the nonstarter lactic acid bac enumeration. On MRS agar, lactobacilli yielded irre light gray and round, creamy colonies, while colonies *thermophilus* were much smaller, pointed, and white the other hand, lactobacilli did not interfere in the count of the starter on skim milk agar, as colonies also different in this culture medium. Lactobacilli yie colonies similar to those on MRS agar, while those *thermophilus* were round and white. Colonies were signed to the *Streptococcus* or *Lactobacillus* genera checking for cell morphology, motility, catalase acti and optimal growth temperature.

All the cheeses showed high initial numbers of starter lactobacilli, which ranged from 5.9 to 7.7 log C g and then remained above 7 log CFU/g during ripe *S. thermophilus* numbers were always ~9 log CFU/g did not decrease during ripening (Table 1).

Changes in starter and nonstarter lactic acid bac counts during the ripening of Pategrás, Cremoso, and milk semihard cheeses are presented in Figure 1. Nonst lactic acid bacteria counts in 10-day-old cheeses made pasteurized milk were above 7 log CFU/g and then creased to as high as 8 log CFU/g during ripening. St tococci remained at values of ~9 log CFU/g for up t



URE 1. *Evolution of starter and nonstarter lactic acid bac-a populations during the ripening of cheeses. Plate counts for nary starter* Streptococcus thermophilus *(hollow signs) and starter lactobacilli (solid signs) in Pategrás (△, ▲), Cremoso ♦), and semihard artisanal cheeses (raw milk) (□, ■).*

r, they increased to 10 log CFU/g in the raw milk cheese r 3 weeks of ripening. The nonstarter lactic acid bacteria nt, in turn, was always higher in raw milk semihard ese, especially after the first month of storage. Coliform eria and yeast and mold counts were always <3 log J/g.

A total of 22 lactobacilli strains was obtained for iden-ation and characterization. A small proportion (12% of the colony total number) was identified as *Enterococcus*. These were isolated and preserved, but no further studies were carried out on them during the present study. Taking into account the results obtained from sugar metabolism patterns and the physiological assays already described (catalase activity, motility, gas production, and cell aggregation), half (11) of the *Lactobacillus* strains were assigned to the *L. casei* group (which includes *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplantarum*, and *L. casei* subsp. *rhamnosus*). From the other isolates, eight belonged to group II of the genus *Lactobacillus* (facultatively heterofermentative), and three belonged to group III (obligately heterofermentative). Strain identification, confirmed by species-specific PCR and DNA sequencing, showed that isolates I72, I81, I84, I85, I86, I88, and I90 were *L. casei*, while isolates I29, I33, I87, I89, and I91 were *L. plantarum*, and isolates I73, I75, I77, and 718 were *L. rhamnosus*. Strains I30, I34, and I48 were *L. curvatus* (group II), while strains I28 and I46 belonged to the species *L. fermentum* (group III). Finally, strain I32 was identified as *L. perolens*.

Cluster analysis of RAPD-PCR patterns of all the studied strains revealed four clusters (clusters 1 to 4) (Fig. 2). Cluster 1 grouped seven strains at a similarity value of ≥85%, including *L. rhamnosus* (four strains), *L. fermentum* (two strains), and *L. perolens* (one strain). These strains came mostly from Tybo and Cremoso cheeses obtained at dairy plants, except for strains I32, I28, and I46 (*L. perolens* and *L. fermentum*), which were isolated from

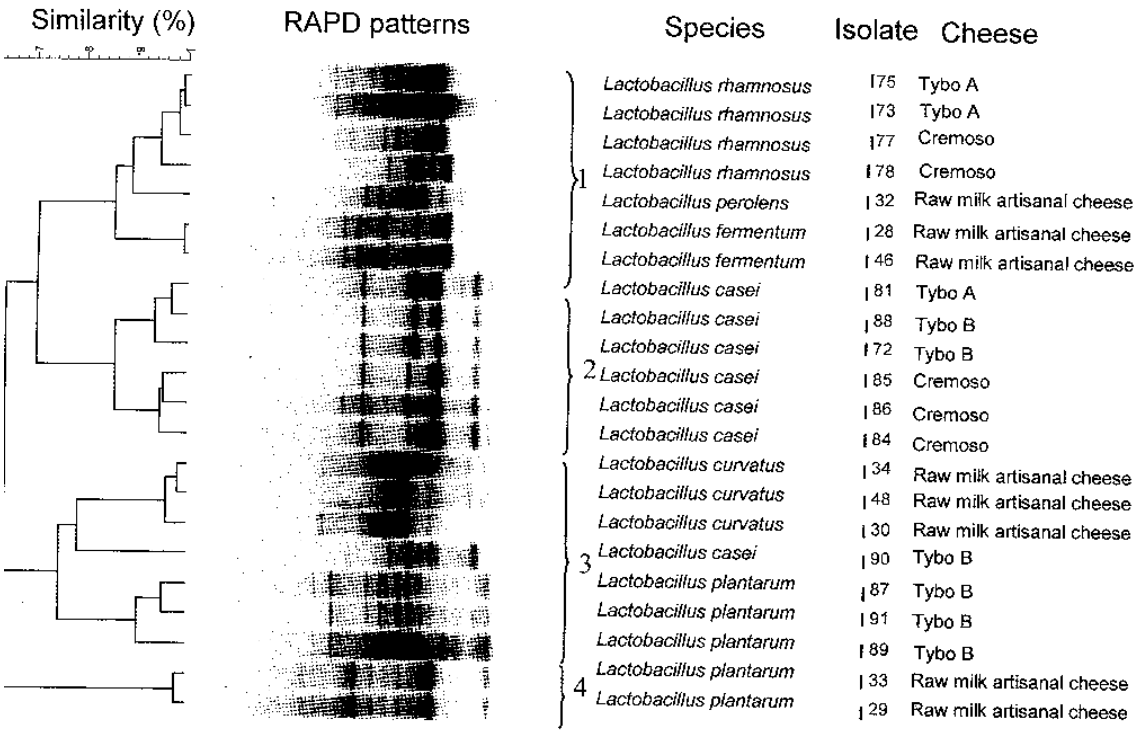


TABLE 2. *Resistance to biological barriers and prebiotic fermentation by nonstarter lactic acid bacteria* Lactobacillus *strains isolat from Argentinean cheeses*

Microorganism	Strain	Resistance to gastric solution (log CFU/ml)[a]	Growth (%) in the presence of[b]: Bile (%): 0.3	0.5	1.0	Lysozyme (ppm): 25	50	100	Prebiotic (2%, wt/vol): Raffinose	Lactulose	Xylitol	Inulin
L. rhamnosus	I73	5.5	44.2	15.5	1.4	96.2	95	87.5	9.6	81.8	6.5	59
	I75	3.2	33.0	32.1	25.0	97.6	76.2	48.8	9.6	87.2	6.6	47.4
	I77	3.8	31.5	18.5	15.2	97.3	75.9	73.2	9.1	69.1	6.6	43.2
	I78	3.4	58.8	47.0	44.1	94.0	88.0	80.0	9.7	100.0	37.8	58.8
L. perolens	I32	7.0	22.5	14.8	8.4	61.5	59.8	56.6	68.6	47.9	9.6	83.3
L. fermentum	I28	4.6	28.5	27.9	13.0	80.5	79.2	74.0	77.1	78.9	1.0	54.5
	I46	4.3	28.5	27.4	13.0	40.7	26.7	10.3	100.0	85.2	0.7	59.0
L. casei	I72	3.8	24.5	10.6	6.7	62.5	60.0	43.7	94.2	50.8	8.1	57.1
	I81	5.6	8.4	6.5	3.5	94.0	78.0	70.0	4.5	90.2	4.7	100.0
	I84	4.9	35.0	26.7	20.8	95.6	89.7	85.3	8.5	8.3	7.1	51.5
	I85	5.8	30.3	16.7	16.7	97.2	95.9	90.5	6.8	87.3	5.7	59.6
	I86	4.4	18.5	10.0	5.8	87.9	67.2	55.2	5.6	96.9	5.0	46.4
	I88	5.8	50.0	23.3	15.0	89.6	75.0	66.7	3.6	87.8	4.1	78.7
	I90	5.8	79.3	50.3	44.7	97.6	96.0	93.2	10.4	96.0	9.1	96.0
L. curvatus	I30	5.1	50.0	39.2	32.4	94.7	91.2	87.7	3.1	84.3	2.4	48.6
	I48	4.9	40.0	33.3	16.7	98.5	73.5	33.8	89.5	100.0	1.2	59.3
	I34	6.2	37.4	29.7	17.6	92.4	81.8	80.1	4.8	80.9	2.6	51.5
L. plantarum	I29	5.1	77.1	54.3	54.3	82.3	72.9	72.9	4.3	89.8	2.6	54.2
	I33	5.1	60.5	55.3	39.5	96.2	92.0	89.0	4.5	82.9	2.6	51.4
	I87	4.9	33.8	18.0	14.4	92.9	88.9	87.1	100.0	89.3	1.6	63.6
	I89	3.9	44.7	25.3	24.7	93.2	91.0	89.3	68.8	100.0	0.2	53.2
	I91	5.8	41.7	37.5	29.2	98.6	97.2	90.3	49.8	91.8	8.6	45.3

[a] Decrease in viable cell counts (log CFU per milliliter) after exposure to a pH 3 solution for 3 h at 37°C (mean of two determinations
[b] With respect to a control (MRS broth).

the semihard raw milk cheese. *L. casei* strains were grouped in cluster 2 (similarity level, 86%) except for strain I90, which was included in cluster 3. Most *L. casei* strains were isolated from cheeses obtained from the same factory. Cluster 3 included strains of *L. curvatus* (three strains), *L. plantarum* (three strains), and *L. casei* (one strain), which were isolated from different samples, at a similarity level of 73%, and showed the highest diversity. These strains came from two cheeses (Tybo B and raw milk cheese). Finally, cluster 4 grouped only two strains of *L. plantarum* isolated from the raw milk cheese, which were quite related (98%).

Pategrás cheese manufactured with pasteurized milk was found to contain thermophilic lactobacilli (*L. delbrueckii* subsp. *bulgaricus*) from the starter employed, which was then confirmed by the cheese makers. Mesophilic lactobacilli counts were obtained on bile-MRS agar, which inhibited *L. delbrueckii* subsp. *bulgaricus*.

Characterization of strains. The resistance of the lactobacilli strains to some biological barriers is summarized in Table 2. Most of the strains showed a decrease in cell counts after incubation in simulated gastric solution that ranged from 4 to 6 log orders. A group of five strains (*L. perolens*) showed the highest loss in cell viability (7 lo orders).

The bile tolerance exhibited by the strains was lowe than that shown against lysozyme. In the presence of 0.3% bile, the growth of most of the strains was between 10 an 50%, compared to the control. Only four strains showed growth rate of >50%. When 1% bile was used, the growt values were <25%, except for four strains, whose percent ages ranged from 32.4 to 54.3%. In the presence of lyso zyme, growth values of >80% were observed for 18 strain (82% of the total) and 12 strains (55% of the total) whe lysozyme at 25 and 100 ppm, respectively, was tested.

The strains showed some differences in their ability t metabolize the prebiotic compounds assayed. Lactulose an inulin were well fermented by the strains: the growth value ranged from 47.9 to 100% and from 43.2 to 100% for lac tulose and inulin, respectively (with the sole exception o *L. casei* I84 in the presence of lactulose). Raffinose wa poorly fermented, as most strains exhibited low growth val ues (<10%), and only eight isolates showed growth value of >50%. Xylitol was the least effective prebiotic among the compounds tested; only *L. rhamnosus* 178 showed growth value of >10%.

Hydrophobicity values, β-galactosidase activity, bil

		solution (log CFU/	Bile (%):			Lysozyme (ppm):			Prebiotic (2%, w/vol):			
nism	Strain	ml)[a]	0.3	0.5	1.0	25	50	100	Raffinose	Lactulose	Xylitol	Inulin
osus	I73	5.5	44.2	15.5	1.4	96.2	95	87.5	9.6	81.8	6.5	59
	I75	3.2	33.0	32.1	25.0	97.6	76.2	48.8	9.6	87.2	6.6	47.4
	I77	3.8	31.5	18.5	15.2	97.3	75.9	73.2	9.1	69.1	6.6	43.2
	I78	3.4	58.8	47.0	44.1	94.0	88.0	80.0	9.7	100.0	37.8	58.8
ns	I32	7.0	22.5	14.8	8.4	61.5	59.8	56.6	68.6	47.9	9.6	83.3
itum	I28	4.6	28.5	27.9	13.0	80.5	79.2	74.0	77.1	78.9	1.0	54.5
	I46	4.3	28.5	27.4	13.0	40.7	26.7	10.3	100.0	85.2	0.7	59.0
	I72	3.8	24.5	10.6	6.7	62.5	60.0	43.7	94.2	50.8	8.1	57.1
	I81	5.6	8.4	6.5	3.5	94.0	78.0	70.0	4.5	90.2	4.7	100.0
	I84	4.9	35.0	26.7	20.8	95.6	89.7	85.3	8.5	8.3	7.1	51.5
	I85	5.8	30.3	16.7	16.7	97.2	95.9	90.5	6.8	87.3	5.7	59.6
	I86	4.4	18.5	10.0	5.8	87.9	67.2	55.2	5.6	96.9	5.0	46.4
	I88	5.8	50.0	23.3	15.0	89.6	75.0	66.7	3.6	87.8	4.1	78.7
	I90	5.8	79.3	50.3	44.7	97.6	96.0	93.2	10.4	96.0	9.1	96.0
us	I30	5.1	50.0	39.2	32.4	94.7	91.2	87.7	3.1	84.3	2.4	48.6
	I48	4.9	40.0	33.3	16.7	98.5	73.5	33.8	89.5	100.0	1.2	59.3
	I34	6.2	37.4	29.7	17.6	92.4	81.8	80.1	4.8	80.9	2.6	51.5
rum	I29	5.1	77.1	54.3	54.3	82.3	72.9	72.9	4.3	89.8	2.6	54.2
	I33	5.1	60.5	55.3	39.5	96.2	92.0	89.0	4.5	82.9	2.6	51.4
	I87	4.9	33.8	18.0	14.4	92.9	88.9	87.1	100.0	89.3	1.6	63.6
	I89	3.9	44.7	25.3	24.7	93.2	91.0	89.3	68.8	100.0	0.2	53.2
	I91	5.8	41.7	37.5	29.2	98.6	97.2	90.3	49.8	91.8	8.6	45.3

se in viable cell counts (log CFU per milliliter) after exposure to a pH 3 solution for 3 h at 37°C (mean of two determinations). spect to a control (MRS broth).

ıihard raw milk cheese. *L. casei* strains were l in cluster 2 (similarity level, 86%) except for)0, which was included in cluster 3. Most *L. casei* were isolated from cheeses obtained from the same Cluster 3 included strains of *L. curvatus* (three . *L. plantarum* (three strains), and *L. casei* (one which were isolated from different samples, at a ty level of 73%, and showed the highest diversity. trains came from two cheeses (Tybo B and raw eese). Finally, cluster 4 grouped only two strains *antarum* isolated from the raw milk cheese, which ite related (98%).

egrás cheese manufactured with pasteurized milk nd to contain thermophilic lactobacilli (*L. del-* subsp. *bulgaricus*) from the starter employed, was then confirmed by the cheese makers. Meso-ctobacilli counts were obtained on bile-MRS agar, nhibited *L. delbrueckii* subsp. *bulgaricus*.

aracterization of strains. The resistance of the lac- strains to some biological barriers is summarized 2. Most of the strains showed a decrease in cell after incubation in simulated gastric solution that from 4 to 6 log orders. A group of five strains (*L. sus* I75, I77, and I78, *L. casei* I72, and *L. plantarum*)wed reductions lower than 4 log orders. Strain I32 (*L. perolens*) showed the highest loss in cell viability (7 log orders).

The bile tolerance exhibited by the strains was lower than that shown against lysozyme. In the presence of 0.3% bile, the growth of most of the strains was between 10 and 50%, compared to the control. Only four strains showed a growth rate of >50%. When 1% bile was used, the growth values were <25%, except for four strains, whose percentages ranged from 32.4 to 54.3%. In the presence of lysozyme, growth values of >80% were observed for 18 strains (82% of the total) and 12 strains (55% of the total) when lysozyme at 25 and 100 ppm, respectively, was tested.

The strains showed some differences in their ability to metabolize the prebiotic compounds assayed. Lactulose and inulin were well fermented by the strains: the growth values ranged from 47.9 to 100% and from 43.2 to 100% for lactulose and inulin, respectively (with the sole exception of *L. casei* I84 in the presence of lactulose). Raffinose was poorly fermented, as most strains exhibited low growth values (<10%), and only eight isolates showed growth values of >50%. Xylitol was the least effective prebiotic among the compounds tested; only *L. rhamnosus* I78 showed a growth value of >10%.

Hydrophobicity values, β-galactosidase activity, bile salt deconjugation ability, and inhibition of pathogenic microorganisms for the studied strains are shown in Table 3.

3 3. *Selected physiological characteristics of nonstarter lactic acid bacteria* Lactobacillus *strains assessed by in vitro experiments*[a]

ganism	Strain	%H[b]	β-gal activity[c]	Deconjugation of bile salt[d]				Inhibition of[e]: *Salmonella* Enteritidis OMS-Ca	*E. coli* V517	*S. aureus* 76	*L. monocytogenes* ATCC 15313
				TC	TDC	GC	GDC				
nnosus	I73	10.9	515	g−	g−	g−	ng−	0.52	0.55	0.49[f]	1.00
	I75	21.7	1,174	g−	g−	g−	ng−	0.33	0.52[f]	1.35[f]	0.90
	I77	26.9	94	g−	g−	g−	ng−	0.97	0.92	1.60	1.30
	I78	21.3	698	g−	wg−	g−	ng−	0.50	0.52	0.91	1.02
olens	I32	82.4	368	g−	g−	g−	ng−	0.29	0.44	1.00[f]	0.66
nentum	I28	85.4	450	g−	g−	g−	ng−	0.45	0.49	0.87[f]	0.75
	I46	35.2	705	g−	g−	g−	ng−	0.33	0.46	0.98[f]	0.62
ei	I72	16.1	350	g−	g−	g−	ng−	0.88	0.57	0.83[f]	1.09
	I81	49.8	453	g−	g−	wg−	ng−	0.96	0.71	0.95[f]	1.14
	I84	22.0	450	g−	g−	g−	ng−	0.85	0.56	0.95[f]	0.85
	I85	55.0	431	g−	g−	g−	ng−	0.56	0.36	1.50[f]	0.55[f]
	I86	40.1	100	g−	g−	g−	ng−	0.70	0.53	0.73[f]	0.58
	I88	27.2	692	g−	ng−	g	ng−	1.02	0.91	1.15[f]	0.97
	I90	20.4	168	wg−	g−	g+	ng−	1.16	1.05	1.60[f]	1.39
vatus	I30	10.3	680	g−	g−	g−	ng−	0.99	0.85	1.57	1.35
	I34	16.4	778	g−	g−	g−	ng−	1.02	0.90	1.50	1.36
	I48	33.1	720	g−	g−	g−	ng−	0.54	0.42[f]	1.37	0.72[f]
ntarum	I29	8.1	430	g−	g−	g−	wg−	0.98	0.81	1.30[f]	1.18
	I33	17.0	458	g−	g	g−	g−	0.98	0.76	1.50[f]	1.11
	I87	24.4	114	g−	g−	g+	g−	1.18	1.10	1.59[f]	1.30
	I89	20.1	115	g−	g−	g+	g−	1.11	0.86	1.30	0.96
	I91	48.7	1,112	g−	g−	g−	g−	0.60	0.43	0.97[f]	0.62

sodium taurocholate; TDC, sodium taurodeoxycholate; GC, sodium glycholate; GDC, sodium glycodeoxycholate.
ent hydrophobicity: mean of three replicates.
alactosidase activity in Miller units.
rowth; ng, no growth; wg, weak growth; −, no bile salt deconjugation; +, bile salt deconjugation.
bition halo diameter (centimeters) − well diameter (1 cm) (means of three determinations).
otes partial growth inhibition.

rophobicity ranged from 8 to 85%. Fourteen strains % of the total) showed values lower than 30%, while ix strains (28% of the total), the hydrophobicity values ed from 33 to 55%. Two strains (*L. fermentum* I28 and *erolens* I32) showed high hydrophobicity values 0%).

β-Galactosidase activity was present in all the strains, values ranging from 94 to 1,174 Miller units. Nine ns showed high β-galactosidase activity, with values er than 500 Miller units. Among them, *L. rhamnosus* and *L. plantarum* I91 showed the highest values, which to more than 1,000 Miller units, and *L. rhamnosus* I78, *ermentum* I46, *L. curvatus* I34, I48, and I30, and *L. i* I88 followed, with about 700 Miller units. *L. rhamus* I73 showed a β-galactosidase activity of 515 Miller s, and the rest of the strains had activities ranging from o 458 Miller units.

A widespread resistance to bile salts (TC, TDC, and was observed among the strains. For GDC, on the rary, only *L. plantarum* strains were capable of growin its presence. On the other hand, bile salts were poorly onjugated by the isolates, as this ability was found in

nella Enteritidis OMS-Ca and *L. monocytogenes* ATCC 15313 were more sensitive than *E. coli* V157, since for the latter, only two lactobacilli strains gave inhibition zones with diameters of >1 cm. *S. aureus* 76 was inhibited by 17 strains (77% of the total) with clear inhibition zones of 1 cm in diameter or larger, even though some of the strains caused only partial inhibition (turbid inhibition zones). Studies regarding the nature of the compounds involved in this antibacterial activity are currently under way in our laboratory.

DISCUSSION

In the cheeses analyzed in this study, initial lactobacilli counts ranged from 5.9 to 7.7 log CFU/g, a level that is unusually high, taking into account previous results on other cheese varieties. It has been shown that lactobacilli grow from very low numbers to 7 to 8 log CFU/g in cheeses made with pasteurized milk, but these counts are usually attempted after the first month of ripening (*(1, 10)*, among others), while we detected values of 6 to 7 log CFU/g in 10-day-old cheeses. Starter bacteria (*S. thermophilus*) counts were about 9 log CFU/g during all ripening times

50). Streptococci and lactobacilli were about 1 log order higher in raw milk cheeses than in pasteurized milk cheeses. Lactic acid bacteria counts in raw milk cheeses were similar to those previously reported *(30, 34, 38, 39).*

Cremoso Argentino cheese, a soft cheese variety without any added microorganism other than *S. thermophilus,* appeared to be an interesting source of adventitious lactobacilli, as nonstarter lactic acid bacteria populations increased similarly in Pategrás, Tybo (semihard), and Cremoso (soft) cheeses, and five lactobacilli strains could be isolated from Cremoso. Most available nonstarter lactic acid bacteria strains have been isolated from Cheddar and other long-ripened cheeses (e.g., *(10, 11, 15, 22)*).

Except for the raw milk cheese, all the cheeses contained only two *Lactobacillus* species: lactobacilli isolated from Tybo A belonged to the species *L. rhamnosus* and *L. casei,* those from Tybo B were *L. casei* and *L. plantarum,* and those from Cremoso were *L. casei* and *L. rhamnosus. L. casei* was found in all the cheeses except for the raw milk cheese, which in turn contained lactobacilli from four different species: *L. plantarum, L. curvatus, L. fermentum,* and *L. perolens.* Most of the isolates were obtained from this raw milk cheese. All the isolated strains belonged to cheese-related lactobacilli species, with the exception of *L. perolens,* which is rarely found in dairy environments *(28).*

In general, it was not possible to correlate groups detected by RAPD-PCR with dairy plants, cheese variety, or cheese making technology, as genotypes showing a high level of similarity belonged to strains isolated from diverse cheeses and factories, except for cluster 4, which contained only two isolates of *L. plantarum* isolated from the raw milk cheese. On the contrary, clusters tended to group strains by species. It is interesting to note that all *L. casei* strains, except for I81, were obtained from cheeses manufactured in the same dairy plant but by different technologies.

The results of the in vitro tests for the assessment of the resistance to biological barriers and other physiological characteristics of the isolated lactobacilli are compatible with potential probiotic properties, taking into account similar studies performed on strains isolated from both intestinal and food sources *(3, 24, 27).* The ability of lactobacilli to survive the passage through the upper gastrointestinal tract must be examined to select lactobacilli for probiotic use *(7).* We found seven strains among the 22 tested lactobacilli that were capable of resisting incubation in simulated gastric solutions in ways similar to or better than the commercial probiotic strains studied previously in comparable conditions *(49).* In addition, most of the strains used in this study were weakly inhibited by bile at 0.3%, and some of them were capable of growth at higher bile concentrations (0.5 and 1%). Lysozyme was, in general, well tolerated by the lactobacilli, which showed considerable growth, even when lysozyme at 100 ppm was present.

Bile salt deconjugation capability has been reported to be a suitable characteristic for the evaluation of lactic acid [illegible] shock protein that enables lactobacilli to survive its exposure to bile *(12).* However, resistance against bile salt toxicity is not necessarily related to deconjugation activity in lactobacilli strains *(29),* and we found that only three isolates were able to deconjugate GC acid salt, while most of them were able to grow in the presence of TC, TDC, GC, and GDC acids. These results agree with previous studies on lactobacilli strains isolated from both intestinal and food sources *(3, 46, 49).*

As for hydrophobicity, this characteristic of lactobacilli cells has been reasonably related to the ability of the microorganism to adhere to epithelial cells *(20).* Several lactobacilli strains studied for this article showed relatively high hydrophobicity values (i.e., above 40%). Other strains showed values of >30%, which are similar to those exhibited by strains marketed as probiotic organisms *(49).*

Improvement in lactose digestion is one of the few probiotic features of lactic acid bacteria that has received general consensus to date *(49).* In our research, we found β-galactosidase activity in all the isolates, which was unlike the findings in previous studies, including those with commercial probiotic strains and lactobacilli from intestinal sources *(46, 49).* In addition—and contrary to previous reports *(19, 40),* which indicated that lactobacilli had relatively low levels of this enzyme—several strains isolated during this study showed high β-galactosidase activity values.

The inhibitory activity shown by the nonstarter lactic acid bacteria strains studied in this article against pathogens by the production of extracellular, diffusible substances is comparable to that previously reported for lactobacilli from intestinal sources against these specific pathogenic strains *(46),* which is significant from two points of view. First, such activity may be related to the safety of the food product, indicating that these strains of lactobacilli would play a beneficial role and contribute to the production of cheeses with low levels of pathogens (or with a low probability of the expression of their pathogenicity). This is particularly important when taking into account that *S. aureus* and *L. monocytogenes* are often responsible for food-poisoning episodes that have involved the consumption of cheese *(6).* Second, the in vitro inhibition of pathogens has been reported as a desirable characteristic of probiotic strains *(3, 46).*

The results of the present study show that the nonstarter lactic acid bacteria lactobacilli isolated from soft and semihard Argentinean cheeses, which belong to the species *L. casei, L. plantarum, L. rhamnosus, L. curvatus, L. fermentum,* and *L. perolens,* demonstrate resistance to biological barriers and physiological characteristics compatible with probiotic properties, which make them suitable for further research during in vivo studies that are aimed at identifying new probiotic organisms. In particular, *L. plantarum* I91, I87, and I89, *L. rhamnosus* I73 and I75, *L. curvatus* I34, and *L. casei* I90 showed the best potential and were

and probiotic characteristics during cheese making and ivo experiments.

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