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Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance

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Abstract

Probiotic characteristics (deconjugation of bile salts, hydrophobicity and β-galactosidase activity) and the resistance to biological barriers (gastric juice and bile salts) of 24 strains of lactic acid starter bacteria (*Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis*) and 24 strains of probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus* and bifidobacteria) were compared. Among the probiotic bacteria tested, *Lactobacillus acidophilus* was the most interesting species since it showed high values of resistance to gastric juice and bile, hydrophobicity and β-galactosidase and bile salts deconjugation activities. *Bifidobacterium bifidum* strains showed the same behavior, although the values of the parameters investigated were slightly lower than those obtained for *Lactobacillus acidophilus*. On the other hand, it was demonstrated that *Lactobacillus delbrueckii* subsp. *bulgaricus* was the lactic acid starter species with the best probiotic characteristics among the starter species assessed. It was resistant to gastric juice and bile, and showed high values for β-galactosidase activity. On the other hand, lactic acid starter bacteria showed hydrophobicity values similar to or higher than those obtained for the strains of the *Lactobacillus casei* . According to the results found, the total probiotic value of a fermented dairy product should take into account not only the intestinal probiotic cultures used in the formulation but also the probiotic contribution of the lactic acid starter microflora.

Keywords: Lactic acid bacteria; Probiotics; Biological barriers

1. Introduction

In 1989, Fuller defined "probiotic" as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance. This has become the most widely accepted definition (Fooks, Fuller, & Gibson, 1999). In this sense, probiotic viability would be a reasonable measure of probiotic activity, but there are situations in which cell viability is not required for probiotic activity such as improved digestion of lactose, some immune system modulation activities and anti-hypertensive effects. In these cases, health beneficial effects have been linked to non-viable cells or to cell components, enzyme activities or fermentation products (Sanders & in't Veld, 1999). There is some debate as to whether the concept of probiotic should include dead microorganisms, or even bacterial fragments (Ziemer & Gibson, 1998). Naidu, Bidlack, and Clemens (1999) introduced the concept of "Probiotic-Active Substance", as a cellular complex of lactic acid bacteria that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independently of viability of lactic acid bacteria.

Nowadays, most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium* (Prasad, Gill, Smart, & Gopal, 1998). However, species belonging to the genera *Lactococcus*, *Enterococcus*, *Saccharomyces* (Salminen & von Wright 1998, Dunne et al., 1999; Sanders & in't Veld, 1999) and *Propionibacterium* (Grant & Salminen, 1998) are also considered as probiotic microorganisms. Even *Streptococcus thermophilus* (Cosson & Deschamps, 1994, Collins, Thornton, & Sullivan, 1998, Naidu et al., 1999, Sreekumar & Hosono, 2000) and *Lb. delbrueckii* subsp. *bulgaricus* (Bezkorovainy, Miller-Catchpole, & Kot, 1997; Naidu et al. 1999; Sreekumar & Hosono, 2000) are considered probiotics. In this

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sense, if we take into account the definitions of "probiotic" given above and the fact that *Lactobacillus* and *Streptococcus* have traditionally been used in fermented dairy products to promote human health (Dunne et al., 1999), it would be interesting to determine the probiotic characteristics of lactic acid starter bacteria in comparison with the traditionally called "probiotic bacteria" since the literature contains many conflicting observations for their proposed benefits (Chou & Weimer, 1999).

The aim of this work was to determine and compare some probiotic characteristics and resistance to biological barriers of lactic acid starter (*S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lc. lactis*) and probiotic (*Lb. casei*, *Lb. rhamnosus*, *Lb. acidophilus*, *Bifidobacterium* sp., *B. bifidum* and *B. longum*) bacteria for establishing the probiotic behavior of their mixtures.

2. Materials and methods

2.1. Strains

For this study, the following strains were used: five commercial strains (A4, A5, A10, DC1 and CC1) and three wild strains (43, 175 and 176, isolated from Argentinian natural milk cultures) of *S. thermophilus*, eight commercial strains of *Lb. delbrueckii* subsp. *bulgaricus* (Ab1, Bb1, Cb1, Db1, Eb3, Eb4, Gb1 and Hb2), eight commercial strains of *Lc. lactis* (13-3, 15-1, 15-4, C12, SL3, SD5, Mo12 and A6), six commercial strains (A3, A9, 08, 53, 5 and CSL) and two collection strains (CNRZ 1881 and CNRZ 1923, obtained from the CNRZ collection, INRA, Jouy-en-Josas, France) of *Lb. acidophilus*, four commercial (A13, A14, LB and BRA) and one collection (CNRZ 1874) strains of *Lactobacillus casei*, three commercial strains of *Lb. rhamnosus* (A15, A16 and LS), three commercial (A12, BBI and Bb12) and one collection strains (ATCC 35914) of *B. bifidum*, three commercial strains of *B. longum* (A1, A7 and BL) and one commercial strain of *Bifidobacterium* sp. (A2). All commercial strains were kindly provided by local industries.

2.2. Culture media and incubation conditions

Lactobacilli and bifidobacteria were cultured in MRS broth (Biokar, Beauvais, France) at 37 °C. Bifidobacteria were incubated in anaerobiosis (GasPak System-Oxoid, Basingstoke, Hampshire, UK). Lactococci and streptococci were grown in Elliker broth (25° and 37 °C, respectively). For lactobacilli and bifidobacteria, cell enumerations were carried out on MRS agar (Biokar, Beauvais, France, 48 h at 37 °C). Bifidobacteria plates were incubated anaerobically. For lactococci and streptococci, the viable counts were performed on Elliker agar (Biokar, Beauvais, France) (48 h at 25 ° and 37 °C, respectively).

To determine β-galactosidase activity, modified-MRS broth (MRS-lac) and modified-Elliker broth (Elliker-lac) were prepared. Thus, all components of MRS and Elliker broth were included except for their original sugars (base broths). Lactose was dissolved in distilled water, sterilized by filtration (filters Millipore, type HA, 0.45 μl, Millipore Corporation, Bedford, MA, USA) and aseptically added to the autoclaved base broths up to a final concentration of 1% (w/v).

2.3. Tolerance to simulated gastric juice

To determine the transit tolerance through simulated gastric juice, the method of Charteris, Kelly, Morelli, and Colline (1998a) was used with slight modifications. Simulated gastric juice was a solution of pepsin (0.3% w/v) and NaCl (0.5% w/v) adjusted to pH 2 and 3. Overnight cultures (30 ml) were centrifuged (6000 × g, 20 min, 5 °C), washed twice in 50 mM K_2HPO4 (pH 6.5) and resuspended in 3 ml of the same buffer. One milliliter of washed cell suspension was harvested by centrifugation (12,000 × *g*, 5 min, 5 °C) and resuspended in 10 ml of gastric solution pH 2 and 3. Total viable counts were performed, as it was detailed above, before and after an incubation period of 3 h at 37 °C. The results were expressed as the difference in these colony counts (log orders CFU ml^{-1}).

2.4. Bile resistance

The ability of the strains to grow in the presence of bile was determined according to the method of Walker and Gilliland (1993) with some modifications. Each strain was inoculated (2% v/v) into MRS broth or Elliker broth with 0.3, 0.5 or 1% (w/v) of bile (Sigma Chemical Co., St. Louis, MO USA). Cultures were incubated at 37 °C (25 °C for lactococci) and, after 24 h, $A_{560\ nm}$ was measured and compared to a control culture (without bile salts). The results were expressed as the percentage of growth ($A_{560\ nm}$) in the presence of bile salts compared to the control.

2.5. Bile salts deconjugation

The ability of the strains to deconjugate bile salts was determined according to the method of Taranto, de Ruiz Holgado, and de Valdez (1995). Bile salt plates were prepared by adding 0.5% (w/v) of sodium salts (Sigma) of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC) and glicodeoxycholic acid (GDC) to MRS agar (for lactobacilli and bifidobacteria) or Elliker agar (for cocci), autoclaved (121 °C, 15 min) and immediately used. The strains were streaked on the media and the plates were anaerobically

(GasPak System-Oxoid) incubated at 37 °C (25 °C for lactococci) for 72 h. The presence of precipitated bile acid around colonies (opaque halo) was considered a positive result.

2.6. Hydrophobicity

The ability of the organisms to adhere to hydrocarbons as a measure of their hydrophobicity, was determined according to method of Perez, Minnaard, Disalvo, and de Antoni (1998) with some modifications. Cultures of the strains were harvested in the stationary phase by centrifugation at 12 000 × *g* for five min at 5 °C, washed twice in 50 mM K_2HPO4 (pH 6.5) buffer and finally resuspended in the same buffer. The cell suspension was adjusted to an A_{560nm} value of approximately 1.0 with the buffer and 3 ml of the bacterial suspensions were put in contact with 0.6 ml of n-hexadecane and vortexed for 120 s. The two phases were allowed to separate for 0 h at 37 °C. 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 n-hexadecane, respectively.

2.7. β-Galactosidase

β-galactosidase activity in whole cells was determined according to the method of Miller (1972) with slight modifications. Overnight cultures of these strains were harvested in the stationary phase by centrifugation at 12 000 × g for 5 min at 5 °C, washed twice in 60 mM $Na_2HPO_4x7H_2O$/40 mM NaH_2PO_4 buffer (pH 7.0) and inoculated (1% v/v) in MRS-lac or Elliker-lac broth. Cultures were incubated at 37 °C (25 °C for lactococci) for 24 h. Cells were harvested and washed twice as previously described and A_{560nm} was adjusted to approximately 1.0 with the same buffer. One milliliter of the cell suspension was permeabilized with 50 μL of toluene/acetone (1:9 v/v) solution, vortexed for 7 min and immediately assayed for β-galactosidase activity. An aliquot of 100 μl of the permeabilized cell suspension was placed in a tube and 900 μl of phosphate buffer and 200 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) (4 mg ml^{-1}) were added. Tubes were placed into a water bath (37 °C) for 15 min (25 °C for lactococci). Then, 0.5 ml of 1M Na_2CO_3 was added to each tube to stop the reaction. Absorbance values at both 420 and 560 nm were recorded for each tube. β-galactosidase activity was calculated (in Miller units) as follows: $1000x[(A_{420}-1.75 \times A2_{560})/(15min \times 1\ ml \times A1_{560})]$, where $A1_{560}$ was the absorbance just before assay and $A2_{560}$ was the absorbance value of the reaction mixture. For the time periods of the assay, the linearity of enzyme assays was previously verified.

Additionally, for lactococci and streptococci strains, *Lb. delbrueckii* subsp. *bulgaricus* Ab1 and Cb1, *Lb. casei* A13, LB and BRA and for *Lb. rhamnosus* A15 and LS the β-galactosidase activity was measured by the method of Gueimonde, Corzo, Vinderola, Reinheimer, and de los Reyes Gavilán (2001) that quantifies the enzyme activity without disrupting the cells. Cultures were aerobically grown overnight at 37 °C in BM broth (10 g tryptone/l, 1.5 g yeast extract/l, 1 ml Tween 80/L, 1.2 g K_2HPO_4/l, 1.5 g sodium acetate/l, 1.2 g diammonium citrate/l, 0.2 g $MgSO_4$/l, 0.17 g bromocresol purple/l, pH 6.6) plus lactose (20 g/l). One milliliter of cell cultures was washed twice, suspended in 3 ml of 60 mM $Na_2HPO_4\times 7H_2O$/40 mM NaH_2PO_4 buffer (pH 7.0) and 2 mL of 0.012 M ONPG were added. The mixture was incubated at 37 °C for 10 min in a water bath. Then, 4 mL of 0.625 M Na_2CO_3 were added to stop the reaction. The contents of each tube were centrifuged at 12,000 × *g* for 5 min to remove the cells. The clear supernatant was recovered and the A_{420} nm was read. The calculation of micrograms of o-nitrophenol (ONP) released was based on the relationship of the $A_{420\ nm}$ to a standard curve. Activity was expressed as mg of ONP released/10 min of incubation.

2.8. Statistical analysis

Experiments were replicated at least three times. Means and standard deviations were calculated using SPSS-PC+ 4.0 software (SPSS Inc., Chicago, IL, USA)

3. Results

The results corresponding to the loss in cell viability after the exposure to simulated gastric juice, growth in the presence of bile salts and their deconjugation, hydrophobicity and β-galactosidase activity for *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lc. lactis* are shown in Table 1. The same kind of results for *Lb. acidophilus*, *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus* and bifidobacteria are displayed in Table 2.

3.1. Tolerance to simulated gastric juice

Lactic acid starter bacteria (Table 1) showed less resistance to simulated gastric juice than probiotic bacteria (Table 2) at both pH values assessed. For the former, all strains (except *Lb. delbrueckii* subsp. *bulgaricus* Eb4 and *Lc. lactis* Mo12) fell at least 6.0 log orders at pH 2. At pH 3, the highest falls in cell viability were, in general, observed for *S. thermophilus*, followed by *Lc. lactis* and *Lb. delbrueckii* subsp. *bulgaricus*. Among probiotic bacteria, *Lb. acidophilus* proved to be the most

Table 1
Probiotic characteristics and resistance to biological barriers for lactic acid starter bacteria (mean ± standard deviation, $n = 3$)

Microorganism/ strain	Resistance to gastric juice[a]		Growth (%) respect to a control, in the presence of bile			Deconjugation of bile salts[b]				H%[c]	β-gal. activity[d]
	pH 3	pH 2	0.3%	0.5%	1%	TC	TDC	GC	GDC		
S. thermophilus											
A4	4.5±0.2	>6.0	6.9±1.2	7.8±1.1	0	–	–	–	–	21.2±2.1	4.5±0.8[e]
A5	5.1±0.1	>6.0	6.4±1.7	4.3±2.0	0	–	–	–	–	16.9±2.0	5.3±0.6 [e]
A10	5.2±0.3	>6.0	5.1±0.9	0	0	–	–	–	–	21.1±1.4	5.1±0.9 [e]
DC1	>6.0	>6.0	4.9±0.7	0	0	wg	wg	wg	–	13.8±2.6	6.7±0.9 [e]
CC1	5.3±0.3	>6.0	5.0±1.1	0	0	wg	–	wg	–	26.6±3.5	4.5±0.7 [e]
43	5.2±0.3	>6.0	4.6±0.2	0	0	–	–	wg	–	17.2±1.0	3.8±0.8 [e]
175	>6.0	>6.0	4.5±0.8	0	0	wg	–	wg	–	12.4±2.6	4.1±0.8 [e]
176	4.0±0.1	>6.0	4.2±0.5	0	0	–	–	wg	–	17.7±4.2	2.9±0.5 [e]
Lb. delbrueckii subsp *.bulgaricus*											
Ab1	2.7±0.1	>6.0	64.0±3.9	48.1±3.6	2.9±1.2	wg	wg	wg	–	27.4±1.1	0
Bb1	3.9±0.3	>6.0	8.5±1.0	6.1±1.0	0	–	–	–	–	15.1±4.2	1750±88
Cb1	3.1±0.2	>6.0	65.2±5.2	36.1±1.5	2.2±0.9	g	wg	wg	–	20.5±2.5	0
Db1	>6.0	>6.0	4.1±0.8	3.8±0.2	2.5±0.8	–	–	–	–	11.0±0.8	2053±25
Eb3	5.0±0.3	>6.0	12.5±1.1	11.2±2.1	0	wg	–	–	–	21.9±2.8	1058±2.7
Eb4	3.2±0.3	4.8±0.6	9.3±1.0	8.4±1.3	0	wg	–	–	–	16.7±2.3	1246±46
Gb1	3.3±0.2	>6.0	18.5±0.6	12.9±0.9	5.1±1.4	wg	g	g	–	10.6±0.7	643±33
Hb2	3.3±0.1	>6.0	8.1±0.4	5.7±1.4	0	wg	–	wg	–	5.86±4.2	518±40
Lc. lactis											
13-3	3.8±0.3	>6.0	76.6±5.1	72.8±6.3	61.3±3.7	g	g	g	g	22.1±4.4	0
15-1	5.3±0.2	>6.0	21.3±1.7	16.5±1.9	0.9±0.2	g	–	wg	–	18.9±1.1	0
15-4	>6.0	>6.0	56.6±2.1	53.4±4.0	44.7±2.3	g	g	g	g	22.1±0.6	0
C12	3.9±0.2	>6.0	39.6±2.7	39.2±2.6	23.1±1.6	g	g	g	g	25.1±0.9	0
SL3	4.5±0.1	>6.0	32.4±3.3	22.9±2.1	13.6±0.9	g	g	g	g	31.3±0.8	0
SD5	3.5±0.4	>6.0	35.9±3.7	34.1±3.6	29.8±1.5	g	g	g	g	19.6±2.2	0
Mo12	3.3±0.8	5.6±0.5	60.1±5.1	52.5±1.8	43.3±2.3	g	g	g	g	14.9±3.4	0
A6	4.3±0.3	>6.0	57.9±4.3	56.5±2.5	36.8±3.0	g	g	g	g	15.5±2.2	0

—: no growth; wg: weak growth; g: growth.

[a] Decrease in viable cell counts (log orders CFU ml^{-1}) after exposure to low pH (3 and 2) solutions during 3 h at 37 °C.

[b] TC: sodium taurocholate, TDC: sodium taurodeoxycholate, GC: sodium glycocholate, GDC: sodium glicodeoxycholate.

[c] Hydrophobicity percentage.

[d] β-galactosidase activity in Miller units.

[e] mg of ONP released/10 min of incubation.

Table 2
Probiotic characteristics and resistance to biological barriers for probiotic bacteria (mean ± standard deviation, $n = 3$)[e]

Microorganism/strain	Resistance to gastric juice [a]		Growth (%) respect to a control, in the presence of bile			Deconjugation of bile salts[b]				H%[c]	β-gal activity[d]
	pH 3	pH 2	0.3%	0.5%	1%	TC	TDC	GC	GDC		
Lc. acidophilus											
A3	1.7±0.3	4.3±0.5	87.4±4.1	83.4±2.1	79.9±1.9	+	++	g	wg	47.9±5.7	954±19
A9	0.9±0.2	3.7±0.4	93.5±3.9	89.5±3.9	79.1±2.3	+	++	g	–	43.8±3.7	822±62
08	0.9±0.3	5.6±0.2	82.0±2.9	82.5±5.0	68.8±3.0	+	+	g	–	38.1±3.6	726±9.0
53	1.7±0.2	4.3±1.8	87.3±5.2	80.4±2.8	79.7±3.1	+	+	g	–	43.0±3.7	675±32
5	1.1±0.1	3.4±0.6	90.3±3.8	98.7±3.4	76.4±2.8	+	++	g	–	50.2±3.3	853±13
CSL	1.1±0.3	4.8±0.1	91.5±4.0	83.4±4.0	77.5±2.9	+	g	g	wg	41.0±4.5	1075±56
CNRZ 1881	3.3±0.8	4.8±0.2	82.0±3.5	81.4±3.7	77.6±3.4	++	+	g	–	50.3±0.2	1301±74
CNRZ 1923	0.7±0.3	5.0±1.2	77.8±3.3	75.1±2.8	45.7±1.3	++	+	g	–	67.8±1.2	1113±14
Lc. casei											
A13	2.7±0.3	> 6.0	82.3±3.7	73.9±4.0	64.1±4.5	g	g	g	–	12.9±2.9	0
A14	5.4±0.3	> 6.0	75.0±6.1	57.7±5.2	37.0±3.6	g	g	g	–	16.9±3.7	395±7.5
LB	3.4±0.2	> 6.0	87.9±3.9	80.0±3.2	62.4±2.0	g	g	g	–	24.1±2.6	0
BRA	4.1±0.3	> 6.0	83.3±2.7	68.3±3.0	61.6±3.8	g	g	g	–	12.0±2.3	0
CNRZ 1874	4.4±0.2	> 6.0	49.2±2.3	49.0±3.0	33.7±2.8	g	g	g	–	14.1±3.5	88.4±8.8
Lc. rhamnosus											
A15	3.2±0.4	> 6.0	66.2±2.3	48.0±1.8	46.1±1.7	g	g	g	–	10.9±3.5	0
A16	3.5±0.3	5.2±1.2	81.9±4.1	64.2±3.1	49.3±0.9	g	g	g	–	19.9±4.0	75±1.3
LS	5.9±0.5	> 6.0	89.6±5.8	84.9±4.5	73.3±5.1	g	g	g	wg	13.7±3.5	0
B. bifidum											
A12	2.3±0.4	3.7±0.1	74.9±2.8	67.6±3.2	41.0±2.8	+	+	g	+	46.7±0.9	480±12
BBI	1.6±0.7	3.3±0.4	57.6±3.5	51.6±2,9	24.1±1.7	g	+	g	+	54.7±2.0	254±16
Bb12	2.3±0.2	4.9±1.1	52.6±5.0	40.6±4.2	24.6±2.1	g	+	g	+	51.8±3.9	502±10
ATCC 35914	2.2±0.2	> 6.0	52.4±4.1	51.6±3.4	18.9±0.9	g	–	g	–	64.7±2.1	426±34
B. longum											
A1	1.4±0.1	> 6.0	15.2±2.0	8.1±1.8	7.1±2.2	g	g	g	g	22.5±0.9	860±52
A7	1.1±0.3	> 6.0	3.6±0.9	4.1±2.0	2.8±2.0	g	g	g	+	27.1±4.8	813±7
BL	0.8±0.1	4.4±1.4	72.7±6.1	67.6±3.9	43.1±5.2	g	+	g	g	28.9±2.3	679±19
B. sp.											
A2	1.4±0.3	4.1±0.1	45.6±3.5	42.4±2.9	26.1±3.0	g	+	g	g	13.6±3.4	147±58

—: no growth; wg: weak growth; g: growth; +: growth and bile salt deconjugation; ++: growth and strong bile salt deconjugation.

[a] Decrease in viable cell counts (log orders CFU ml^{-1}) after exposure to low pH (3 and 2) solutions during 3 h at 37 °C.

[b] TC: sodium taurocholate, TDC: sodium taurodeoxycholate, GC: sodium glycocholate, GDC: sodium glicodeoxycholate.

[c] Hydrophobicity percentage.

[d] β-galactosidase activity in Miller units.

[e] mg of ONP released/10 min of incubation.

resistant species, with losses in cell viability ranging from 3.4 to 5.0 log orders and from 0.7 to 3.3 log orders at pH 2 and 3, respectively. For bifidobacteria, these values ranged from 3.3 to more than 6.0 log orders at pH 2 and from 0.8 to 2.3 log orders at pH 3 while the strains of *Lb. casei* and *Lb. rhamnosus* showed losses in cell viability higher than 5.0 log orders at pH 2 and from 2.7 to 5.9 log orders at pH 3.

3.2. Bile resistance

The presence of bile salts was more inhibitory for lactic acid starter bacteria than for probiotic organisms. Among the former, *S. thermophilus* was the most sensitive species since 0.5% of bile inhibited most of the strains. On the other hand, *Lc. lactis* strains proved to be quite resistant even in the presence of 1% of the bile salts. All the probiotic bacteria strains were more or less resistant to 1% bile salts. In general terms, *Lb. acidophilus* was the species that grew better (> 69% for 1% bile, except for *Lb. acidophilus* CNRZ 1923) followed by the species belonging to *Lb. casei* and *Lb. rhamnosus* (from 3 to 73%, 1% bile) and bifidobacteria (from 3% to 43%, 1% bile).

3.3. Bile salts deconjugation

Except for lactococci, lactic acid starter bacteria showed sensitivity to the presence of conjugated bile salts. On the other hand, probiotic bacteria showed, in general, to be tolerant to these compounds and some strains were even able to deconjugate them. Regarding individual bile salts, sodium glycodeoxycholate completely inhibited the growth of all the strains of *S. thermophilus* and *Lb.* delbrueckii subsp. *bulgaricus*. The other bile salts assayed were less inhibitory towards these species since they allowed a weak growth of some strains. Except for *Lc. lactis* 15-1, all lactococci strains were able to grow in the presence of individual bile salts but no bile salt deconjugation was observed. Except for bifidobacteria, sodium glycodeoxycholate was determined to inhibit most of the probiotic strains examined. The other salts allowed the growth of the three probiotic species. Deconjugation activity was observed, for *Lb. acidophilus*, on sodium taurocholate and sodium taurodeoxycholate and, for some strains of bifidobacteria, on sodium taurodeoxycholate and glycodeoxycholate. Deconjugation activity was not observed for the strains belonging to *Lb. casei* and *Lb. rhamnosus*.

3.4. Hydrophobicity

Lactic acid starter species showed quite similar values for hydrophobicity among them and in any case lower than 32%. In general, these values were lower than those found for probiotic bacteria. However, it was interesting to see that the values obtained for most of lactic acid starter strains were higher than those found for the strains of *Lb. casei* and *Lb. rhamnosus*. For probiotic strains, the hydrophobicity values found ranged from 38.1 to 67.8% (*Lb. acidophilus*) from 13.6 to 64.7% (bifidobacteria) and from 10.9 to only 24.1% for the strains of *Lb. casei* and *Lb. rhamnosus*.

3.5. β-galactosidase

The β-galactosidase activity was present in higher number of probiotic strains compared to lactic acid starter cultures. No β-galactosidase activity was detected for lactococci. Among *Lb. delbrueckii* subsp. *bulgaricus* strains these values ranged from 0 to 2053 Miller units. The β-galactosidase activity of *S. thermophilus* strains was only detected and quantified by the method of Gueimonde et al. (2001). For probiotic bacteria, absence of β-galactosidase or low values of this enzyme were detected for *Lb. casei* and *Lb. rhamnosus* by both methods assessed, while for bifidobacteria, β-galactosidase activity values ranged from 147 to 860 Miller units. The highest values (ranging from 675 to 1301 Miller units) were obtained for *Lb. acidophilus* strains.

4. Discussion

Nowadays, lactic acid starter bacteria are widely used in combination with probiotic (*Bifidobacterium*, *Lactobacillus*) bacteria to manufacture fermented dairy products. In Argentina, commercial strains of *Lb. acidophilus*, *Lb. casei*, *Lb. rhamnosus* and *Bifidobacterium* are added to fermented milks, like yoghurts (manufactured with *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* cultures) and probiotic cheese (manufactured with *Streptococcus* and *Lactococcus* cultures) (Vinderola, Bailo, & Reinheimer, 2000a, Vinderola, Prosello, Ghiberto, & Reinheimer, 2000b).

Due to their poor ability to survive through the passage of the stomach and the gastrointestinal tract, lactic acid starter bacteria were usually not thought to be probiotics (IDF, 1999). However, more recent criteria (Naidu et al., 1999, Lee, Nomoto, Salminen, & Gorbach, 1999) have included *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* as members of the probiotic organisms list since these are able to release, among other compounds, enzymes (as β-galactosidase) that improve the digestion of nutrients in the intestine as well as modulate immune responses, that also play a positive role in human health. According to this, the total probiotic value of a fermented dairy product should take into account not only the intestinal probiotic cultures used for the microbiological formulation but also the probiotic contribution of the lactic acid starter microflora. In this

work, some probiotic characteristics and the resistance to biological barriers were compared for lactic acid starter and probiotic cultures used in combination by the Argentinian dairy industry.

About 2.5l of gastric juice at a pH of approximately 2.0 is secreted each day in the stomach (Charteris et al., 1998a), which causes the destruction of most microorganisms ingested (Kimoto, Ohmomo, Nomura, Kobayashi, & Okamoto, 2000). In this sense, resistance to human gastric transit is an important selection criterion for probiotic microorganisms (Charteris, Kelly, Morelli, & Collins, 1998b). The intrinsic resistance to acid (and bile) of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* is poor (Charteris et al., 1998a). In this work *S. thermophilus* strains tested showed to have, in fact, very poor survival when exposed to low pH solutions. Better survival was shown by some strains of *Lb. delbrueckii* subsp. *bulgaricus*, but only at pH 3 (except the strain Eb4 that was resistant also at pH 2). Although some authors have shown the contrary (Klijn, Weerkamp, & de Vos, 1995), it has been generally assumed that *Lactococcus* strains do not survive passage through the digestive system (Kimoto, Kurisaki, Tsuji, Ohmomo, & Okamoto, 1999). In our work, the loss of cell viability in simulated gastric juice for lactococci strains was higher than that reported by Kimoto et al. (2000). The ability of probiotic bacteria to survive the passage through the stomach was reported to be variable and strain-dependent (Clark, Cotton, & Martin, 1993; Charteris et al. 1998a, Zavaglia, Kociubinsky, Pérez, & de Antoni, 1998; Chung, Chun, & Ji, 1999). Out of 200 strains of lactobacilli and bifidobacteria, Prasad et al. (1998) were able to select only a few strains with satisfactory acid resistance. In the present study, the tolerance to gastric transit was also observed to be variable among the strains tested. Contrary to Xanthopoulos, Litopoulu-Tzanetaki, and Tzanetakis (2000), that reported a better survival for *Lb. casei* than for *Lb. acidophilus* ones, in this study, the latter species survived better than the other microorganisms studied. Finally, for *B. bifidum* strains the values found for cellular death in low pH solutions were within the ranges previously reported (Zavaglia et al., 1998; Chung et al., 1999) but higher than those reported by Marteau, Minekus, Havennar and Huis in't Veld (1997) and Clark et al. (1993). According to Dunne et al. (1999), bifidobacteria strains proved to be significantly less acid resistant than *Lb. acidophilus*. However, from our results, it was observed that bifidobacteria were as resistant as *Lb. acidophilus*, at pH 2.

The relevant physiological concentrations of human bile range from 0.3% (Dunne et al. 1999) to 0.5% (Zavaglia et al. 1998). In this sense, it is generally considered necessary to evaluate the ability of potentially probiotic bacteria to resist the effects of bile acids (Collins et al., 1998, Dunne et al., 1999) not only because it is a selection criterion (Mattila-Sandholm, Matto, & Saarela, 1999; Ouwehand, Kirjavainen, Shortt, & Salminen, 1999), but also because lactobacilli and bifidobacteria have been shown to exhibit a strain variation in their tolerance to bile salts (Charteris et al., 1998b, Xanthopoulos et al., 2000, Zarate, Pérez Chaia, González, & Oliver, 2000). The intrinsic resistance to bile salts found in this study was poor for *S. thermophilus*, moderate for *Lb. delbrueckii* subsp. *bulgaricus* and satisfactory for lactococci strains. This fact, together with a moderate gastric transit tolerance, could partially guarantee the survival in the gastrointestinal tract of *Lb. delbrueckii* subsp. *bulgaricus* and *Lactococcus*, as it was previously reported for the latter by Klijn et al. (1995). Bile tolerance is considered to be an important characteristic of *Lb. acidophilus* (Walker & Gilliland 1993). Among the probiotic strains tested in this study, *Lb. acidophilus* showed, in fact, the highest bile salts tolerance, followed by the other probiotic lactobacilli and bifidobacteria. According to Marteau et al. (1997) a low bile resistance would not be a disadvantage for probiotic strains since the intracellular β-galactosidase might be released from the cells by lysis during passage through the gastrointestinal tract or, at least, permeabilized cells might be necessary in order for an efficient lactose hydrolysis to occur in the small intestine (Mustapha, Jiang, & Savaiano, 1997). Thus, species with low gastric and small intestine transit tolerance may be essential for in vivo lactose digestion (Charteris et al., 1998b). However, this hypothesis has met opposite opinions since other works proved that the most bile-tolerant strains significantly contributed to reduce symptoms of lactose intolerance (Zárate et al., 2000).

The inhibition of common intestinal bacteria has been related to the presence of free (deconjugated) bile acids rather than conjugated ones (De Smet, Van Hoorde, Vande Woestyne, Cristianes, & Verstraete, 1995, Grill, Cayuela, Antoine & Schneider, 2000). The deconjugation activity would play a role in maintaining the equilibrium of the gut microflora (Taranto et al., 1995; Taranto, Gonzalez de Llano, Rodríguez, Pesce de Ruiz Holgado, & Font de Valdez, 1996). It has been also suggested that bile salt hydrolase (BSH) enzyme might be a detergent shock protein that enables lactobacilli to survive the intestinal bile stress (De Smet et al., 1995). Another phenomenon related to the presence of the deconjugation activity is the reduction of serum cholesterol (Corzo & Gilliland 1999a). In this sense, bile salt deconjugation is a desirable property when selecting a strain to be used as a dietary adjunct. In our work, no deconjugation activity was observed for lactic acid starter bacteria and for *Lb. casei* and *Lb. rhamnosus*, but BSH activity was observed for bifidobacteria and *Lb. acidophilus* strains. While *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were, in general, inhibited by bile salts, *Lc. lactis* proved to be resistant to them since it

was able to grow in the medium containing 0.5% of any of the bile salts assessed. Contrary to the findings of Brashears, Gilliland, and Buck (1998) and Hashimoto et al., (2000) who determined the ability of strains of *Lb. casei* to deconjugate sodium taurocholate, in our study, no deconjugation activity of this type was observed for the strains of this group. As it had been previously reported by Brashears et al. (1998), Corzo and Gilliland (1999b) and Hashimoto et al. (2000), all our strains of *Lb. acidophilus* were able to deconjugate sodium taurocholate and sodium taurodeoxycholate although no deconjugation of sodium glycocholate was observed. It was suggested (Tanaka, Doesburg, Iwasaki, & Mierau, 1999) that strains with BSH activity come from an intestinal environment in which they are exposed to bile salts. However, the fact that not all strains of gastrointestinal origin have BSH shows that bacteria without this enzyme can either survive in this environment or survive the passage through it. Some strains of a particular species do not possess the deconjugation activity while others do (as in our study, the deconjugation of sodium glicodeoxycholate was observed only for some strains of bifidobacteria). The fact that some strains were able to grow in the presence of conjugated bile salts while they were not able to deconjugate them is in accordance with the hypothesis that the capacity to express bile salt hydrolase is not related to the capacity to resist the toxicity of conjugated bile salts (Moser & Savage, 2001).

Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells (Savage, 1992). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Rosenberg, Gutnick, & Rosenberg, 1980; Kiely & Olson, 2000). This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Naidu et al., 1999). The determination of microbial adhesion to hexadecane as a way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely & Olson, 2000). In our work, the highest values of hydrophobicity were found for the *Lb. acidophilus* and *Bifidobacterium* strains, while lower values were obtained for the strains of *Lb. casei* and *Lb. rhamnosus*, in coincidence with previously reported values (Kiely & Olson, 2000). The hydrophobicity values observed for *B. bifidum* strains were lower than those previously reported by Zavaglia et al. (1998). It was interesting to see that hydrophobicity values for lactic acid starter bacteria were similar or higher than those obtained for some probiotic ones (*Lb. casei*, *Lb. rhamnosus*, *B. longum* and *B.* sp.).

It is generally accepted that most fermented milks improve lactose digestion (IDF, 1999; Ouwehand et al., 1999), which is one of the few well established probiotic effects of lactic acid bacteria identified to date (Charteris et al., 1998b). In our work, no β-galactosidase was detected for lactococci and, in general, for the strains of *Lb. casei* and *Lb. rhamnosus*. It is interesting to remark that for streptococci, the method of Miller (1972) was not able to allow the contact between the enzyme and the substrate (ONPG). The highest values of this enzyme were found in *Lb. delbrueckii* subsp. *bulgaricus*, followed by *Lb. acidophilus* and bifidobacteria, contrary to previous reports (IDF, 1999) that established that lactobacilli generally have a lower content of β-galactosidase than bifidobacteria. The values found for *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus* are in the range of values previously reported (Shah & Jelen, 1990, Ibrahim & O' Sullivan, 2000) while for bifidobacteria they were significantly smaller (Ibrahim & O'Sullivan, 2000). In a previous work (Smart, Pillidge, & Garman, 1993), where the distribution-in lactic acid starter and probiotic bacteria- of β-galactosidasa and phospho-β-galactosidase was examined, the former enzyme was found in bifidobacteria, *S. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. acidophilus*, for some strains of lactococci phospho–galactosidase activity was also reported, while for *L. casei* only this was found. These variations observed among strains further emphasize the importance of selecting appropriate strains for use as dietary adjuvants (Hughes & Hoover, 1995, Fernandez Murga, Hebert, Savoy de Giori, & Font de Valdez, 1997).

The results of this work indicate that, for intestinal probiotic and starter lactic acid bacteria, the probiotic characteristics and resistance to biological barriers are very variable among species and even among strains belonging to the same species. So, an adequate strain selection must be carried out to manufacture probiotic dairy products. When both groups of bacteria are used together, the total "probiotic value" of foods might take into account the eventual probiotic contribution of the starter lactic acid strains involved.

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