Journal of Dairy Research (2012) **79** 119–127. © *Proprietors of Journal of Dairy Research 2012*
doi:10.1017/S0022029911000884

Probiotic potential of *Lactobacillus* spp. isolated from Brazilian regional ovine cheese

Stela Maris Meister Meira, Virginia Etges Helfer, Renata Voltolini Velho, Fernanda Cortez Lopes and Adriano Brandelli*

Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brazil

Received 1 June 2011; accepted for publication 1 October 2011

Twelve *Lactobacillus* isolates from Brazilian starter-free ovine cheeses were evaluated for their probiotic potential. The strains were identified by 16S rDNA sequencing as *Lactobacillus plantarum* (7), *Lb. brevis* (2), *Lb. casei* (2) and *Lb. parabuchneri* (1). All strains showed variable resistance to gastric juices and relative tolerance to pancreatin and bile salts. Only five strains of *Lb. plantarum* could not deconjugate the sodium salt of taurodeoxycholic acid. Autoaggregation ability after 24 h was above 50% and hydrophobicity was higher than 60% for most strains. All lactobacilli could inhibit linolenic acid oxidation, except *Lb. parabuchneri* strain, whereas none of them could scavenge DPPH radical. β-Galactosidase activity ranged from 47·7 to 2503 Miller units. Inhibition of food pathogens *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Salmonella typhimurium* was demonstrated and the production of organic acids could be associated with this effect. The *Lactobacillus* strains from Brazilian regional ovine cheese showed interesting functional characteristics, mainly the strains *Lb. brevis* SM-B and *Lb. plantarum* SM-I. Both presented high acid tolerance. In addition, *Lb. brevis* SM-B also displayed remarkable antioxidant activity and *Lb. plantarum* SM-I was the highest β-galactosidase producer, exhibited high autoaggregation and hydrophobicity properties.

Keywords: Probiotic, ovine cheese, lactic acid bacteria, *Lactobacillus*.

The relationship between diet and health/disease, as well as economic interests of industry and health care costs, stimulate the development of new products in which health-promoting functional properties can exceed the sensory and nutritional concerns of foods. Increasingly, the concept of functional foods has to be taken into account, which implies the ability to beneficially influence body functions in order to improve the state of well-being and health, reducing the risk of diseases (Granato et al. 2010). Probiotic microbial strains are among the functional components with properties to alter intestinal microbiota and/or immune barriers, moreover the probiotic-containing foods are generally perceived as 'safe' and 'natural' (Begley et al. 2006).

Reduction and prevention of diarrhoea from different origins, improvement of the intestinal microbial balance by antimicrobial activity, and alleviation of lactose intolerance symptoms are among the scientifically established and/or clinically proved health effects of probiotics (Parvez et al. 2006; Vasiljevic & Shah, 2008). Furthermore, some studies have shown that certain lactic acid bacteria possess antioxidant activity (Lin & Chang, 2000; Kullisaar et al. 2002).

Lactobacillus species are desirable members of intestinal tract microbiota and increasingly being explored as probiotics in various food products. Since not all lactobacilli can confer health benefits to the host, the isolation and characterization of strains with specific and well-characterized activities appears appropriate not only to improve the quality and functional properties of probiotic products but also to advance both applied and fundamental science in the probiotics area (Pan et al. 2009). Resistance to the manufacturing process of the carrier food and subsequently to the gastrointestinal ecosystem are required (Valerio et al. 2006). The ability of probiotic bacteria to reach, survive and persist in the environment in which it is intended to act have to be assessed to ensure optimal functionality and expression of health promoting physiological functions (Zanoni et al. 2008). The selected bacteria should be tolerant to low pH, proteolytic enzymes and bile salts, and should be prevalent in the upper digestive

*For correspondence; e-mail: abrand@ufrgs.br

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

tract (Huang & Adams, 2004). For adhesion purposes and colonization, surface hydrophobicity and aggregation properties may be relevant (Collado et al. 2008; Xu et al. 2009).

The autochthonous microbial content of cheeses may play a relevant role in the sensory characteristics, the safety hazards and potential health benefits of that product (Pisano et al. 2007; Nespolo et al. 2010). Few studies have focused on probiotic characteristics of lactobacilli isolated from ewe's cheeses (Caldini et al. 2008; Corsetti et al. 2008). The aim of the present work was to investigate the probiotic potential of novel *Lactobacillus* strains isolated from ovine cheese manufactured in the South Region of Brazil.

Materials and Methods

Isolation of Lactobacillus *strains*

Starter-free raw ovine milk cheeses (60 d ripening) were collected from a commercial cheese plant in Southern Brazil. Samples (25 g) were diluted in 225 ml of 1 g l^{-1} peptone water and homogenized in a blender. Serial 10-fold dilutions were done and inoculated on Man–Rogosa–Sharpe (MRS) agar (Himedia). After anaerobic incubation at 30 °C for 72 h, typical colonies were randomly selected from countable plates, and then transferred to the MRS broth. The LAB selection included those strains showing Gram-positive staining, rod-shaped, catalase-negative and reaching pH below 5·0 in MRS broth. The strains denominated as LCN 35 and LCN 39 were isolated previously from Brazilian regional ovine cheese by Nespolo & Brandelli (2010), but not yet identified and characterized concerning probiotic effect. The standard strains *Lactobacillus plantarum* ATCC 8014 and *Lb. fermentum* ATCC 9338 were used for comparison purposes. All bacteria were stored at −20 °C in 20% (v/v) glycerol and propagated twice on MRS broth before use.

Identification of lactic acid bacteria

Strains were identified by 16S rDNA sequence analysis as described elsewhere (Lisboa et al. 2006). The BLAST algorithm was used to search for homologous sequences in GenBank and the software CLUSTAL W version 1.8 was used for sequences alignment (Thompson et al. 1994).

Haemolysis

The lactobacilli strains were streaked on blood agar plates and incubated for 48 h at 30 °C (Maragkoudakis et al. 2006). Strains that produced green-hued zones around the colonies (α-haemolysis) or did not produce any effect on the blood plates (γ-haemolysis) were classified as non haemolytic. Strains displaying blood lysis zones around the colonies were considered haemolytic (β-haemolysis).

Acid and bile salts tolerance

The tolerance to upper gastrointestinal tract was evaluated in simulated gastric and intestinal juices (Huang & Adams, 2004). Bacterial cells with 24 h incubation in MRS broth were harvested by centrifugation (12 000 ***g*** for 5 min at 4 °C), washed twice in 10 mmol l^{-1} phosphate buffer pH 7·0 and suspended in 5 g l^{-1} NaCl solution. An aliquot of 0·2 ml cellular suspension was incubated at 37 °C in the presence of 1·0 ml of simulated juices. Viable cell counts were determined at initial time and at 180 min for gastric transit tolerance and after 240 min for intestinal transit tolerance.

Simulated gastric juice was prepared fresh daily containing 3 mgpepsin ml^{-1} (Sigma Co., St Louis, MO, USA), 5 g NaCl l^{-1} and acidified with HCl to pH 3·0, 2·5 and 2·0. Otherwise, simulated intestinal juice consisted of 1 mgpancreatin ml^{-1} (Merck, Darmstadt, Alemanha), 5 g NaCl l^{-1} and adjusted to pH 8·0, with or without 5 g l^{-1} of a 1:1 mixture of sodium cholate and sodium deoxycholate (Sigma Co., St Louis, MO, USA). Both solutions were sterilized by filtering through 0·22 µm membranes (Millipore, Bedford, USA).

Bile salt deconjugation

Overnight cultures were spotted (5 µl) onto MRS agar plates containing 0·5% (w/v) sodium taurodeoxycholate (Sigma Co., St Louis, MO, USA) and 0·37 g $CaCl_2$ l^{-1} and incubated anaerobically for 72 h at 37 °C (Pinto et al. 2006). Opaque white colonies or colonies with precipitated zones were considered to indicate deconjugation of bile salts. MRS agar plates without supplementation were used as controls.

Autoaggregation and hydrophobicity

The *in vitro* properties of autoaggregation and hydrophobicity were performed as a preliminary screening of adhesive probiotic strains (Collado et al. 2008). Lactobacilli strains were incubated at 30 °C for 24 h in MRS broth and cells were harvested by centrifugation, washed twice and suspended in 10 mmol l^{-1} phosphate buffer pH 7·0 to 0·25 ± 0·02 absorbance at 600 nm.

The autoaggregation assay was performed as follows: the cellular suspensions were incubated at 37 °C and absorbance values at 600 nm of the upper layer were measured at different interval times (2, 16, 20 and 24 h). The results were expressed as percentage: $1 - (A_{600\,nm}$ of upper suspension/ $A_{600\,nm}$ of total bacterium suspension) × 100.

In the hydrophobicity evaluation, xylene was used to determine bacterial adhesion to the hydrocarbon. Then, a cellular suspension volume (3 ml) was thoroughly mixed by vortexing for 60 s with 400 µl xylene. After 2 h at 37 °C, the aqueous phase was carefully removed and absorbance at 600 nm was measured. The hydrophobicity was reported as adhesion percentage: $[(A_0 - A)/A] \times 100$, where A_0 and A are the absorbance before and after extraction with xylene, respectively.

β-Galactosidase activity

The β-galactosidase (β-gal) activity was determined according to Vinderola & Reinheimer (2003). Briefly, isolates were

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

cultivated in lactose-MRS broth and the cells washed and permeabilized with 1:9 (v/v) toluene:acetone by vortexing. After, the bacterial suspensions were incubated with *o*-nitrophenyl-β-D-galactopyranoside (ONPG: Sigma Co., St Louis, MO, USA) at 37 °C for 15 min and the reaction was stopped with 1 mol l^{-1} Na_2CO_3. Absorbance values were measured at 420 and 560 nm and results expressed as Miller units as follows:

$$\beta\text{-gal activity} = 1000 \times [(A_{420} - 1{\cdot}75 \times A2_{560})/ (15\,\text{min} \times 1\,\text{ml} \times A1_{560})]$$

where $A1$ was the absorbance just before assay and $A2$ was the cell density of the reaction mixture.

Antioxidant activity assays

Antioxidant activity of whole cells (optical density of 2·5 at 600 nm) was determined by two different assays. Linolenic acid peroxidation assay was carried out according to Kullisaar et al. (2002) based on the ability of bacteria to inhibit linolenic acid oxidation, expressed in percentage. In addition, DPPH radical scavenging capacity of the isolates was verified using the method of Brand-Williams et al. (1995). An aliquot of 0·05 ml of cellular suspensions was transferred to test tubes with 2 ml of freshly prepared 60 µmol l^{-1} DPPH methanolic solution. After 45 min, the mixture was centrifuged and the scavenging activity was measured spectrophotometrically by the decrease in absorbance at 517 nm. Likewise, blank value was determined by using 1·15 g KCl l^{-1} solution.

Screening for antimicrobial activity

Detection of antibacterial activity against *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 1901, *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14078, was performed by the agar spot test (Jacobsen et al. 1999). A 5 µl volume of overnight cultures was spotted onto MRS agar plates and incubated anaerobically to develop colonies at 30 °C for 24 h. Then, 10 ml Brain Heart Infusion (BHI, Oxoid) soft agar (7·5 g l^{-1}) inoculated with indicator strain (10^7 CFU ml^{-1}) was poured onto plates and incubated aerobically at 37 °C for 24 h. The inhibition results (mm) were calculated subtracting the diameter of the inhibition zone from the diameter of lactobacilli colony.

The same agar spot method was further used to test the activity of cell free supernatants, obtained from overnight cultures grown in MRS broth at 30 °C. After centrifuging the culture at 12 000 ***g*** for 5 min at 4 °C, the supernatants were obtained, neutralized or not with 5 mol l^{-1} NaOH and filtered through 0·22 µm membranes. In addition, well diffusion and microtrite plate assays were carried out as described by Maragkoudakis et al. (2006).

Heat sensitivity was checked by heating supernatants at 121 °C for 15 min and later antimicrobial activity was evaluated by microtitre plate assay. Supernatants were also treated with 2 mgtrypsin ml^{-1} for 60 min at 37 °C before testing for antimicrobial activity (Bizani & Brandelli, 2002). MRS broth (pH 6·5) and MRS broth acidified with lactic acid and HCl solutions to the pH value reached normally by *Lactobacillus* strains (pH about 4·0) were used as controls.

Results

Identification of lactic acid bacteria and haemolysis

A total of 12 lactobacilli strains from ovine cheese manufactured in the South Region of Brazil were considered in this study, two of which were previously isolated (Nespolo & Brandelli, 2010). The molecular identification of the isolates showed *Lb. plantarum* as the predominant species (7 strains), followed by *Lb. brevis* (2 strains) and *Lb. casei* (2 strains). Additionally, one strain was identified as *Lb. parabuchneri* (Table 1). None of the *Lactobacillus* strains were positive for haemolytic reaction (all of them γ-haemolytic).

Resistance to simulated gastric and intestinal juices and bile salt deconjugation

Lb. brevis SM-B was the most resistant strain at pH 3·0 and pH 2·5 in pepsin-containing simulated gastric juices (reduction of 0·3 and 2·3 log, respectively), followed by *Lb. plantarum* SM-I, LCN 35, SM-M and SM-C strains (Table 1). In contrast, *Lb. brevis* SM-A exhibited the highest decrease in viable counts at pH 2·5. *Lb. parabuchneri, Lb. plantarum* SM-5 and *Lb. casei* isolates also showed significant loss of viability in both treatments. None of the strains could survive at pH 2·0 for 3 h, only *Lb. brevis* SM-B could be detected above detection limit (>1·7 CFU ml^{-1}) after 1 h (data not shown) in this condition.

All strains showed little reduction in viability after 240 min in simulated pancreatic juice (Table 1). However, with the addition of 0·5% unconjugated bile salts (sodium cholate and deoxycholate) a more pronounced decrease was often observed, especially for *Lb. casei* SM-G (3·8 log orders). In relation to the ability to deconjugate the sodium deoxycholate, only *Lb. plantarum* strains SM-C, SM-I, SM-N, LCN 35 and LCN 39 could not hydrolyse this bile salt (data not shown).

Autoaggregation and hydrophobicity

Autoaggregation values increased through the incubation time (Table 2). Although the behaviour of strains could not be clearly denoted in the first 2 h, it was more apparent from 16 h until final incubation period. Most strains showed more than 50% aggregation after 24 h, highlighting *Lb. casei* SM-H as the most autoaggregative strain (79·8%) and *Lb. parabuchneri* SM-L as the lowest autoaggregative strain (36·3%).

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

Table 1. Resistance to biological barriers for *Lactobacillus* strains isolated from cheese of Southern Brazil and for the reference strains (mean ± SD)

		Resistance to gastric juice†		Resistance to intestinal juice‡	
Strains	Initial population*	pH 3	pH 2·5	Without bile salts	With 0·5% bile salts
Lb. plantarum					
SM-5	8·8 ± 0·3	6·0 ± 0·3	2·9 ± 0·1	8·8 ± 0·3	8·1 ± 0·3
SM-C	8·6 ± 0·1	7·9 ± 0·8	4·9 ± 0·6	8·2 ± 0·3	7·7 ± 0·7
SM-I	8·9 ± 0·3	8·1 ± 0·4	6·1 ± 0·5	8·4 ± 0·3	7·8 ± 0·4
SM-M	8·9 ± 0·2	7·9 ± 0·5	5·6 ± 0·8	8·3 ± 0·2	8·3 ± 0·2
SM-N	9·1 ± 0·4	7·2 ± 0·6	4·9 ± 0·2	8·0 ± 0·2	7·9 ± 0·6
LCN 35	8·8 ± 0·4	7·9 ± 0·5	5·9 ± 0·7	8·9 ± 0·4	8·8 ± 0·1
LCN 39	8·9 ± 0·1	7·1 ± 0·4	3·7 ± 0·8	8·9 ± 0·2	8·9 ± 0·4
ATCC 8014	8·8 ± 0·3	7·5 ± 0·2	5·0 ± 0·7	8·2 ± 0·1	7·8 ± 0·3
Lb. brevis					
SM-A	8·6 ± 0·1	5·4 ± 0·5	< 1·7	8·4 ± 0·1	8·2 ± 0·3
SM-B	8·9 ± 0·3	8·6 ± 0·1	6·6 ± 0·2	8·6 ± 0·1	8·5 ± 0·2
Lb. casei					
SM-G	8·9 ± 0·3	6·4 ± 0·1	2·2 ± 0·1	8·9 ± 0·1	5·1 ± 0·7
SM-H	8·5 ± 0·1	4·9 ± 0·5	2·8 ± 0·7	8·3 ± 0·2	7·6 ± 0·4
Lb. parabuchneri SM-L	8·9 ± 0·2	5·3 ± 0·3	2·5 ± 0·3	8·3 ± 0·7	7·7 ± 0·2
Lb. fermentum ATCC 9338	8·4 ± 0·1	6·3 ± 0·3	5·3 ± 0·4	8·4 ± 0·1	7·1 ± 0·7

*Means of viable cell counts (log orders CFU/ml) at the initial time of the treatments
†Viable cell counts (log orders CFU/ml) after exposure to low pH (3 and 2·5) solutions during 3 h at 37 °C
‡Viable cell counts (log orders CFU/ml) after exposure to pancreatin solutions (with and without 0·5% bile salts) during 4 h at 37 °C

Table 2. *In vitro* adhesion properties of *Lactobacillus* strains isolated from cheese of Southern Brazil and for the reference strains (mean ± SD)

	Autoaggregation (%)				
Strains	2 h	16 h	20 h	24 h	Hydrophobicity (%)
Lb. plantarum					
SM-5	21·7 ± 1·8	45·7 ± 0·5	51·1 ± 1·0	55·4 ± 2·5	70·6 ± 6·7
SM-C	19·9 ± 1·0	65·1 ± 1·2	68·7 ± 10	72·6 ± 2·1	75·4 ± 7·1
SM-I	16·7 ± 3·5	66·1 ± 1·0	71·9 ± 2·3	74·7 ± 3·6	76·5 ± 4·0
SM-M	18·1 ± 8·1	55·2 ± 6·3	59·4 ± 5·7	66·6 ± 5·0	60·1 ± 4·1
SM-N	16·4 ± 2·5	36·4 ± 2·2	40·0 ± 1·4	45·1 ± 3·1	62·2 ± 1·5
LCN 35	28·6 ± 3·0	59·0 ± 0·4	62·2 ± 0·9	67·0 ± 1·6	33·6 ± 1·2
LCN 39	22·6 ± 0·8	50·9 ± 3·1	53·2 ± 4·0	56·3 ± 1·2	69·3 ± 3·4
ATCC 8014	19·8 ± 2·7	49·7 ± 4·6	51·0 ± 5·8	56·2 ± 5·4	34·9 ± 7·5
Lb. brevis					
SM-A	18·4 ± 2·6	38·9 ± 4·6	45·2 ± 2·4	52·3 ± 5·0	88·0 ± 2·1
SM-B	14·7 ± 4·3	37·8 ± 1·8	41·9 ± 2·8	43·9 ± 3·2	34·6 ± 2·7
Lb. casei					
SM-G	15·7 ± 4·0	35·4 ± 2·9	39·3 ± 3·2	43·3 ± 4·4	15·2 ± 7·0
SM-H	32·2 ± 3·9	70·1 ± 2·1	75·2 ± 1·7	79·8 ± 2·6	51·7 ± 3·3
Lb. parabuchneri SM-L	11·3 ± 3·6	29·0 ± 3·4	31·9 ± 3·2	36·3 ± 0·6	29·4 ± 5·8
Lb. fermentum ATCC 9338	28·6 ± 6·8	58·2 ± 2·5	60·9 ± 4·9	66·9 ± 6·1	75·1 ± 5·1

The degree of hydrophobicity (Table 2) was high for *Lb. plantarum* isolates, except for LCN 35. *Lb. brevis* SM-A was the most hydrophobic (88%), while *Lb. casei* SM-G was the isolate showing the lowest hydrophobicity (15·2%).

β-Galactosidase activity

To determine the enzymatic activity, lactose was used as inducer, replacing the glucose in the MRS medium. Results revealed strains with different values of enzymatic

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

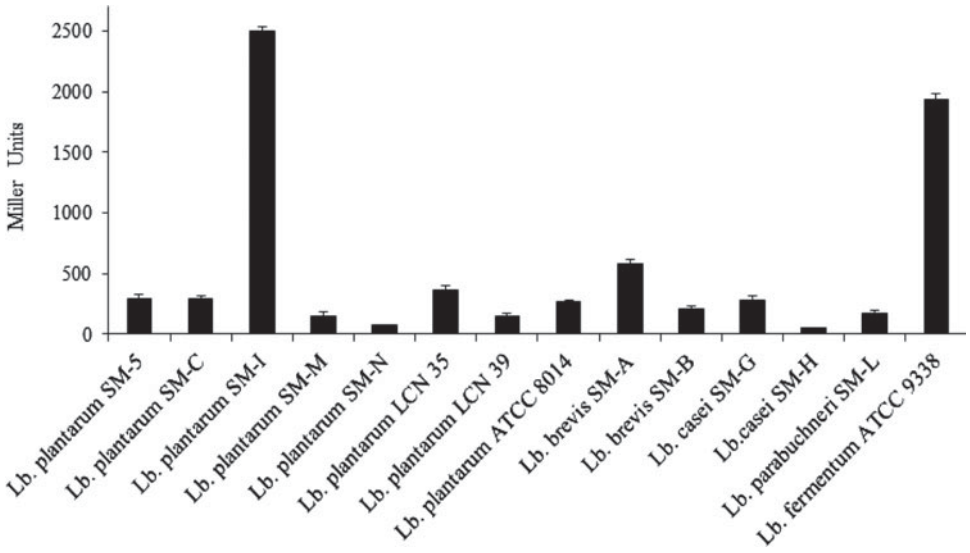

Fig. 1. β-Galactosidase activity of *Lactobacillus* strains isolated from ovine milk cheese and the reference strains. Values are the means of three independent determinations ± SEM.

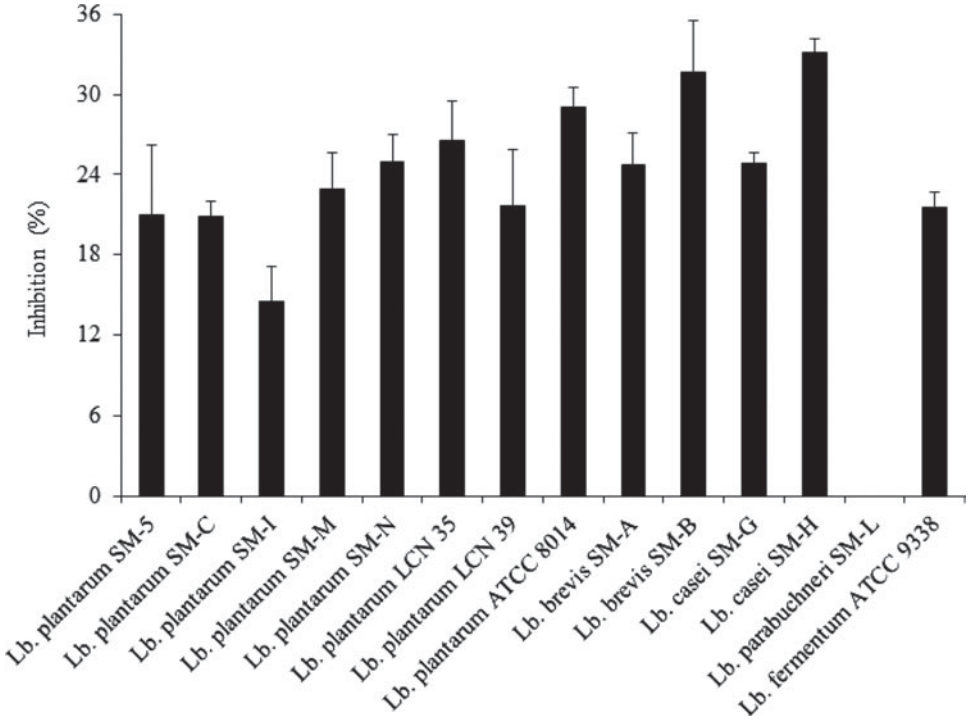

Fig. 2. Inhibition of linolenic acid peroxidation by *Lactobacillus* strains isolated from ovine milk cheese and the reference strains. Values are the means of three independent determinations ± SEM.

activity (Fig. 1), *Lb. casei* SM-H having the lowest value (47·7 Miller units) and values higher than 500 Miller units were only observed for *Lb. fermentum* ATCC 9338 and *Lb. plantarum* SM-I (1941 and 2503 Miller units, respectively).

Antioxidant activity

Linolenic acid oxidation assay was used to determine the capability of the strains to inhibit lipid peroxidation. The results are summarized in Fig. 2, indicating that all

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

Table 3. Antagonistic activity by colonies of *Lactobacillus* strains isolated from cheese of Southern Brazil and for the reference strains (mean ± SD)

	Zone of inhibition (mm)				
Strains	*Listeria monocytogenes* ATCC 7644	*Bacillus cereus* ATCC 14579	*Staphylococcus aureus* ATCC 1901	*Escherichia coli* ATCC 8739	*Salmonella typhimurium* ATCC 14078
Lb. plantarum					
SM-5	16·4 ± 1·9	15·5 ± 0·7	13·5 ± 2·0	13·3 ± 1·5	12·7 ± 2·0
SM-C	14·4 ± 0·5	13·3 ± 2·4	12·4 ± 1·4	11·7 ± 1·5	15·3 ± 1·3
SM-I	16·6 ± 1·0	14·7 ± 2·3	13·7 ± 1·9	8·4 ± 2·0	12·4 ± 1·8
SM-M	16·2 ± 1·9	17·1 ± 2·0	14·0 ± 2·0	9·0 ± 1·7	12·9 ± 2·0
SM-N	16·9 ± 0·8	19·3 ± 1·5	14·5 ± 1·8	9·8 ± 1·8	15·3 ± 1·4
LCN 35	16·7 ± 1·2	13·0 ± 0·8	20·0 ± 1·4	11·0 ± 1·0	13·1 ± 1·0
LCN 39	18·3 ± 1·5	15·5 ± 0·9	18·7 ± 0·5	12·7 ± 0·6	12·5 ± 0·8
ATCC 8014	15·8 ± 1·7	14·2 ± 1·8	14·4 ± 1·6	12·4 ± 2·0	14·3 ± 2·0
Lb. brevis					
SM-A	13·2 ± 2·1	9·7 ± 2·0	13·5 ± 0·7	12·0 ± 1·4	12·2 ± 1·8
SM-B	14·0 ± 1·9	13·6 ± 1·9	13·9 ± 1·2	11·2 ± 1·7	17·3 ± 1·3
Lb. casei					
SM-G	13·3 ± 2·0	12·9 ± 1·9	14·3 ± 1·4	15·3 ± 2·1	11·6 ± 1·1
SM-H	11·4 ± 2·0	10·5 ± 2·2	13·3 ± 0·5	13·7 ± 2·2	11·1 ± 2·0
Lb. parabuchneri SM-L	10·0 ± 1·0	5·5 ± 0·7	8·5 ± 2·1	6·0 ± 0·5	5·7 ± 1·0
Lb. fermentum ATCC 9338	13·2 ± 2·2	14·7 ± 1·1	11·4 ± 1·6	11·6 ± 1·2	10·6 ± 1·9

strains, except *Lb. parabuchneri* SM-L, possess antioxidant ability ranged from 14·5 to 33·1%. In contrast, none of the strains showed scavenging capacity of DPPH radical (data not shown).

Antimicrobial activity

The results of antibacterial activity demonstrated that all strains inhibited both Gram positive and Gram negative bacteria (Table 3). In general, *Lb. plantarum* strains showed greater inhibitory zones against most of the pathogens tested. *Lb. casei* strains showed better inhibition of *Esch. coli*, in comparison with other tested strains. *Lb. parabuchneri* strain had little inhibitory effect against all tested pathogens.

When supernatants were evaluated by agar spot test and well diffusion assay, no inhibition was observed, even when non-neutralized supernatants were analysed. When microtitre plate assay was performed, only supernatants at pH about 4·0 (normally reached by the *Lactobacillus* strains) could inhibit the pathogens tested, even after heat treatment or trypsin incubation (data not shown). In the same way, controls acidified at pH 4·0 with HCl and lactic acid also inhibit pathogenic strains by that method.

Discussion

Lactobacillus species isolated from ovine cheese manufactured in the South Region of Brazil were identified by 16S rDNA. Other reports on ovine cheeses also found the species *Lb. plantarum, Lb. casei* and *Lb. brevis* (Sánchez et al. 2006; Majhenic et al. 2007; Navidghasemizad et al. 2009). *Lb. parabuchneri* is an obligatory heterofermentative occasionally isolated from cheeses (Coton et al. 2008), though it is reported as a frequently isolated species from Civil cheese (Sengül, 2006) and the predominant species in Tulum cheese samples (Sengül & Cakmakci, 2003).

The determination of haemolytic activity is considered a safety aspect for the selection of probiotic strains (FAO/WHO, 2002) and none of the *Lactobacillus* tested exhibited it. Also, the resistance to gastric acidity and to bile salts are the two currently most used *in vitro* tests according to the guidelines for the evaluation of probiotics in food (FAO/WHO, 2002).

In most *in vitro* assays, pH 3·0 has been chosen to evaluate resistance to gastric transit due to substantial decrease in the viability of strains at pH 2·0 or lower (Vinderola & Reinheimer, 2003). Our results also showed that acid tolerance is strain-specific and, as described by Zanoni et al. (2008), it is a trait of individual strains and strongly affected by experimental conditions. Thus, a direct comparison with other studies is difficult to be established. Nevertheless, similar studies often demonstrated that lactobacilli had increased sensitivity at pH values below 3·0 (Maragkoudakis et al. 2006; Ortu et al. 2007). Guo et al. (2009) emphasized that variation in the acid tolerance might be related to the difference in H^+-ATPase activity, which controls the intracellular H^+ concentration, thereby maintaining pH homeostasis and cell viability (Corcoran et al. 2005). The quantity and physiological state of the

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

bacteria influence the survival during the gastrointestinal transit. Likewise, when probiotic bacteria are delivered from food, the buffering capacity of food matrix constitutes a major factor affecting stomach pH and could enhance gastric tolerance, as demonstrated by several studies (Pinto et al. 2006; Valerio et al. 2006; Guglielmotti et al. 2007).

The presence of bile salts and pancreatin in the small intestine is another biological barrier for probiotic bacteria survival and colonization. The concentration of bile salts and specific bacteria properties are possible factors influencing the effect of bile salts on growth. Our results are in agreement with other studies for propionic bacteria (Huang & Adams, 2004) and lactobacilli (Maragkoudakis et al. 2006). In contrast, some reported strains are able to grow in presence of high concentration of bile salts (Vinderola & Reinheimer, 2003; Ortu et al. 2007).

Bile salt hydrolase activity (BSH) is not always related to high tolerance to bile salts (Moser & Savage, 2001; Guglielmotti et al. 2007). Cholic acid is one of the most common free bile acids in the intestine and one of the mechanisms of resistance is the capacity to deconjugate bile salts (Vinderola & Reinheimer, 2003; Guglielmotti et al. 2007; Mathara et al. 2008). Moreover, deconjugation of bile salts may play a role in maintaining the equilibrium of the gut microflora, and could be used for selecting cholesterol-reducing strains, but conversion of primary to secondary procarcinogenic bile salts in the intestine is a potential risk associated with BSH activity (Begley et al. 2006; Pinto et al. 2006; Mathara et al. 2008). The two strains of *Lb. casei*, SM-G and SM-H, can deconjugate taurodeoxycholic acid, differing from strains of the same species described by Vinderola & Reinheimer (2003), Guglielmotti et al. (2007) and Mathara et al. (2008).

The autoaggregation and hydrophobicity properties can be useful to select potentially probiotic bacteria (Collado et al. 2008; Xu et al. 2009). Hydrophobicity could enable interaction with organic mucin layer of the gut (Mathara et al. 2008), confer a competitive advantage for bacterial maintenance in the gastrointestinal tract (Vinderola & Reinheimer, 2003) and imply a potential capacity to activate the gut immune response (Vinderola et al. 2008). Sometimes, highly hydrophobic cell surfaces are associated with self-aggregation ability (Collado et al. 2008; Xu et al. 2009). Thus, the correlation between these properties was shown by SM-G, SM-L and SM-B isolates, which presented the lowest values for both self-aggregation and hydrophobicity. These two properties are likely to be correlated with adhesion ability to epithelial cells and mucosal surfaces (Collado et al. 2008; Martins et al. 2009; Xu et al. 2009). However, lactobacilli adhesion is a complex phenomenon dependent not only on achievement of an adequate mass through aggregation and physicochemical properties (like hydrophobicity) but also on more specific mechanisms involving chemical composition of both intestinal and microbial cell surfaces (Collado et al. 2008; Martins et al. 2009).

All strains tested exhibited β-gal activity. This enzyme is produced by most lactobacilli with both hydrolase and transglycosilase activities, advantageous from technological and health point of views. Thus, the discovery of new strains producing high level of β-gal has gained importance for potential applications as probiotic cultures in dairy industry or as producers of the prebiotic ingredients galacto-oligosaccharides (Ibrahim & O'Sullivan, 2000; Ustok et al. 2010).

Comparable results are described by Vinderola & Reinheimer (2003), with higher values obtained for the *Lb. delbrueckii* subsp. *bulgaricus* starter strain (2053 Miller units) and for *Lb. acidophilus* probiotic strain (1301 Miller units), while low values or absence of β-gal were detected in *Lb. casei* strains. Cebeci & Gürakan (2003) also found strong variation among *Lb. plantarum* strains. In the present work, several strains showed higher β-gal activity in comparison with *Lb. plantarum* ATCC 8014. These results are in contrast with those reported by Pinto et al. (2006) for lactobacilli isolated from faeces and fermented products.

Lactic acid bacteria possess different antioxidative mechanisms such as reduced glutathione, superoxide dismutase, NADH oxidase, NADH peroxidase, thiol compounds, metal ion chelating ability, scavenge of reactive oxygen species and reducing activity. These protective capabilities result in antioxidant properties by some lactobacilli and potentially provide additional dietary source of antioxidants or probiotic bacteria able to reduce oxidative stress (Kullisaar et al. 2002; Zanoni et al. 2008; Wang et al. 2009).

The same total antioxidant assay used in this work carried out in different groups of intestinal lactobacilli revealed the highest values for obligate homofermentative lactobacilli, whereas for facultative and obligate heterofermentative lactobacilli the activity was strain-specific (Annuk et al. 2003). Zanoni et al. (2008) described an effective antioxidant activity by intact cells of *Lb. plantarum* LP1 and *Streptococcus thermophilus* Z57, with 33·1 and 33·8% inhibition of linolenic acid oxidation, respectively. These values are similar to those observed for *Lb. brevis* SM-B and *Lb. casei* SM-H, the most antioxidative strains of this study. However, the scavenging capacity of DPPH radical was not observed (data not shown). Wang et al. (2009) reported *in vitro* scavenging capacity against the DPPH radical in a dose dependent manner by whole cells of a strain of *Lb. fermentum*. Then, whereas no DPPH scavenging ability was detected in our strains, they could inhibit linolenic acid peroxidation and further experiments are needed to substantiate antioxidant activity.

Lactic acid bacteria may exert a protective barrier to intestinal cells by preventing colonization of pathogenic microorganisms. Antimicrobial metabolites include certain organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, carbon dioxide, diacetyl, acetaldehyde, reuterin, acetoin, fatty acids, other low molecular mass compounds and bacteriocins (Holzapfel et al. 1995; De Vuyst & Leroy, 2007). Moreover, competitive exclusion for nutrients and adhesion sites and stimulation or modulation of immune

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

responses reinforces the antimicrobial activity (Servin, 2004).

The *Lactobacillus* strains isolated from ovine cheese and the reference strains could inhibit all pathogens tested by direct antagonism. The non-neutralized culture supernatants were inhibitory when the microtitre plate assay was performed, but not using agar spot test and well diffusion assay. The inhibitory activity observed may be associated with the production of organic acids and low pH, since no inhibition was observed when the pathogens were grown either in the presence of neutralized supernatants or with the MRS broth at pH 6·5. Similar situation was described by Maragkoudakis et al. (2006), who found that none of the supernatants of 29 lactobacilli strains, at pH 6·5 or 4·5, inhibited the growth of *Esch. coli, Sal. typhimurium* and *Helicobacter pylori*, using either the spot-on-lawn or the well diffusion assay. Only when microtitre plate assay was performed, the authors described the inhibition of pathogens by supernatants at pH 4·5 and by the control of MRS at pH 4·5.

Production of organic acids, the most important and best characterized antimicrobials from LAB, is the probable mechanism of inhibition of the strains analysed in this work. It is interesting because the ability to adequately acidify the intestine is a beneficial property derived from probiotic lactobacilli to create a hostile environment for pathogens.

The results of this work suggest that ripened cheeses elaborated with raw ewe's milk in Southern Brazil are interesting sources for isolation of bacterial strains with differentiated functional traits. Notably, probiotic characteristics are very variable among strains, even those belonging to the same species, and none of strains possess all the desired properties. However, isolates that demonstrate the greatest number of biological and functional attributes together are *Lb. brevis* SM-B, with the better acid tolerance and remarkable antioxidant activity, and *Lb. plantarum* SM-I, the highest β-gal producer and displaying higher autoaggregation, hydrophobicity and acid tolerance properties.

This work was supported by CNPq and CAPES (Brazil).

References

Annuk H, Shchepetova J, Kullisaar T, Songisepp E, Zilmer M & Mikelsaar M 2003 Characterization of intestinal lactobacilli as putative probiotic candidates. *Journal of Applied Microbiology* **94** 403–412

Begley M, Hill C & Gahan CGM 2006 Bile salt hydrolase activity in probiotics. *Applied and Environmental Microbiology* **72** 1729–1738

Bizani D & Brandelli A 2002 Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. *Journal of Applied Microbiology* **93** 512–519

Brand-Williams W, Cuvelier ME & Berset C 1995 Use of a free radical method to evaluate antioxidant activity. *LWT Food Science and Technology* **28** 25–30

Caldini G, Trotta F, Corsetti A & Cenci G 2008 Evidence for *in vitro* anti-genotoxicity of cheese non-starter lactobacilli. *Antonie van Leeuwenhoek* **93** 51–59

Cebeci A & Gürakan C 2003 Properties of potential probiotic *Lactobacillus plantarum* strains. *Food Microbiology* **20** 511–518

Collado MC, Meriluoto J & Salminen S 2008 Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology* **226** 1065–1073

Corcoran BM, Stanton C, Fitzgerald GF & Ross RP 2005 Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology* **71** 3060–3067

Corsetti A, Caldini G, Mastrangelo M, Trotta F, Valmorri S & Cenci G 2008 Raw milk traditional Italian ewe cheeses as a source of *Lactobacillus casei* strains with acid-bile resistance and antigenotoxic properties. *International Journal of Food Microbiology* **125** 330–335

Coton M, Berthier F & Coton E 2008 Rapid identification of the three major species of dairy obligate heterofermenters *Lactobacillus brevis, Lactobacillus fermentum* and *Lactobacillus parabuchneri* by species-specific duplex PCR. *FEMS Microbiology Letters* **284** 150–157

De Vuyst L & Leroy F 2007 Bacteriocins from lactic acid bacteria: production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology* **13** 194–199

FAO/WHO 2002 *Guidelines for the evaluation of probiotics in food*. London, Ontario: Food and Agriculture Organization of the United Nations and World Health Organization Working Group Report, 1–11

Granato D, Branco GF, Nazzaro F, Cruz AG & Faria JAF 2010 Functional foods and nondairy probiotic food development: Trends, concepts, and products. *Comprehensive Review of Food Science and Food Safety* **9** 292–302

Guglielmotti DM, Marcó MB, Golowczyc M, Reinheimer JA & Quiberoni LQ 2007 Probiotic potential of *Lactobacillus delbrueckii* strains and their phage resistant mutants. *International Dairy Journal* **17** 916–925

Guo Z, Wang J, Yan L, Chen W & Liu X 2009 *In vitro* comparison of probiotic properties of *Lactobacillus casei* Zhang, a potential new probiotic, with selected probiotic strains. *LWT Food Science and Technology* **42** 1640–1646

Holzapfel WH, Geisen R & Schillinger U 1995 Ecological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology* **24** 343–362

Huang Y & Adams MC 2004 *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. *International Journal of Food Microbiology* **91** 253–260

Ibrahim SA & O'Sullivan DJ 2000 Use of chemical mutagenesis for the isolation of food grade β-galactosidase overproducing mutants of bifidobacteria, lactobacilli and *Streptococcus thermophilus*. *Journal of Dairy Science* **83** 923–930

Jacobsen C, Rosenfeldt N, Hayford A, Møller P, Michaelsen K, Pærregaard A, Sandström B & Tvede M 1999 Screening of probiotic activities of forty seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology* **65** 4949–4956

Kullisaar T, Zilmer M, Mikelsaar M, Vihalemm T, Annuk H, Kairane C & Kilk A 2002 Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology* **72** 215–224

Lin M & Chang F 2000 Antioxidative effect of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356. *Digestive Disease Science* **45** 1617–1622

Lisboa MP, Bonatto D, Bizani D, Henriques JAP & Brandelli A 2006 Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. *International Microbiology* **9** 111–118

Majhenic AC, Lorberg PM & Rogelj I 2007 Characterisation of the *Lactobacillus* community in traditional Karst ewe's cheese. *International Journal of Dairy Technology* **60** 182–190

Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B & Tsakalidou E 2006 Probiotic potential of *Lactobacillus* strains isolated from dairy products. *International Dairy Journal* **16** 189–199

Martins FS, Silva AA, Vieira AT, Barbosa FHF, Arantes RME, Teixeira MM & Nicoli JR 2009 Comparative study of *Bifidobacterium animalis, Escherichia coli, Lactobacillus casei* and *Saccharomyces boulardii* probiotic properties. *Archives of Microbiology* **191** 623–630

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press

Mathara JM, Schillinger U, Guigas C, Franz C, Kutima PM, Mbugua SK, Shin HK & Holzapfel WH 2008 Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. *International Journal of Food Microbiology* **126** 57–64

Moser SA & Savage DC 2001 Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Applied and Environmental Microbiology* **67** 3476–3480

Navidghasemizad S, Hesari J, Saris P & Nahaei MR 2009 Isolation of lactic acid bacteria from Lighvan cheese, a semihard cheese made from raw sheep milk in Iran. *International Journal of Dairy Technology* **62** 260–264

Nespolo CR & Brandelli A 2010 Production of bacteriocin-like substances by lactic acid bacteria isolated from regional ovine cheese. *Brazilian Journal of Microbiology* **41** 1009–1018

Nespolo CR, Correa APF, Ritter AC & Brandelli A 2010 Comparison of Fascal cheese produced with natural, commercial or autochthonous cultures. *International Journal of Dairy Technology* **63** 387–395

Ortu S, Felis GE, Marzotto M, Deriu A, Molicotti P, Sechi LA, Dellaglio F & Zanetti S 2007 Identification and functional characterization of *Lactobacillus* strains isolated from milk and Gioddu, a traditional Sardinian fermented milk. *International Dairy Journal* **17** 1312–1320

Pan X, Chen F, Wua T, Tang H & Zhao Z 2009 The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. *Food Control* **20** 598–602

Parvez S, Malik KA, Kang SA & Kim HY 2006 Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology* **100** 1171–1185

Pinto MGV, Franz CMAP, Schillinger U & Holzapfel WH 2006 *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. *International Journal of Food Microbiology* **109** 205–214

Pisano MB, Fadda ME, Deplano M, Corda A, Casula M & Cosentino S 2007 Characterizarion of Fiore Sardo cheese manufactured with the addition of autochthonous cultures. *Journal of Dairy Research* **74** 255–261

Sánchez I, Seseña S, Poveda JM, Cabezas L & Palop L 2006 Genetic diversity, dynamics, and activity of *Lactobacillus* community involved in traditional processing of artisanal Manchego cheese. *International Journal of Food Microbiology* **107** 265–273

Sengül M 2006 Microbiological characterization of Civil cheese, a traditional Turkish cheese: microbiological quality, isolation and identification of its indigenous lactobacilli. *World Journal of Microbiology and Biotechnology* **22** 613–618

Sengül M & Cakmakci S 2003 Characterization of natural isolates of lactic acid bacteria from Erzincan (Savak) Tulum cheese. *Milchwissenschaft* **58** 510–513

Servin AL 2004 Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *FEMS Microbiology Reviews* **28** 405–440

Thompson JD, Higgins DG & Gibson TJ 1994 CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22** 4673–4680

Ustok FI, Tari C & Harsa S 2010 Biochemical and thermal properties of β-galactosidase enzymes produced by artisanal yoghurt cultures. *Food Chemistry* **119** 1114–1120

Valerio F, Bellis PD, Lonigro SL, Morelli L, Visconti A & Lavermicocca P 2006 *In vitro* and *in vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastrointestinal tract. *Applied and Environmental Microbiology* **72** 3042–3045

Vasiljevic T & Shah NP 2008 Probiotics – From Metchnikoff to bioactives. *International Dairy Journal* **18** 714–728

Vinderola CG & Reinheimer JA 2003 Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. *Food Research International* **36** 895–904

Vinderola G, Capellini B, Villarreal F, Suárez V, Quiberoni A & Reinheimer JA 2008 Usefulness of a set of simple *in vitro* tests for the screening and identification of probiotic candidate strains for dairy use. *LWT Food Science and Technology* **41** 1678–1688

Wang AN, Yi XW, Yu HF, Dong B & Qiao SY 2009 Free radical scavenging activity of *Lactobacillus fermentum in vitro* and its antioxidative effect on growing-finishing pigs. *Journal of Applied Microbiology* **107** 1140–1148

Xu H, Jeong HS, Lee HY & Ahn J 2009 Assessment of cell surface properties and adhesion potential of selected probiotic strains. *Letters in Applied Microbiology* **49** 434–442

Zanoni S, Pompei A, Cordisco L, Amaretti A, Rossi M & Matteuzzi D 2008 Growth kinetics on oligo- and polysaccharides and promising features of three antioxidative potential probiotic strains. *Journal of Applied Microbiology* **105** 1266–1276

https://doi.org/10.1017/S0022029911000884 Published online by Cambridge University Press