International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/1471-0307.12360

ORIGINAL RESEARCH

Wild *Lactobacillus* strains: Technological characterisation and design of Coalho cheese lactic culture

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To design a specific lactic culture for the controlled manufacture of coalho cheese, 13 Lactobacillus rhamnosus*, two* Lactobacillus fermentum *and one* Lactobacillus plantarum *strains isolated from artisanal coalho cheeses were identified and characterised. Two* Lb. rhamnosus*, one* Lb. plantarum *and one* Lb. fermentum *were selected and grouped in pairs designing four different culture formulations that demonstrated a good performance in cheesemaking experiments at pilot scale. Further studies to adjust the balance of strains used are necessary to attain adequate sensorial and technological attributes as expected for artisanal cheeses.*

Keywords Adjunct culture, *Lactobacillus rhamnosus*, Lactic acid bacteria.

INTRODUCTION

Among regional dairy products of Brazilian Northeast, coalho cheese is one of the most traditional. It is a semihard cheese, produced mostly with raw milk and has a relevant socioeconomic importance, as its production and marketing is the main source of income for many families. Coalho cheese is also a cultural identity element, demanded by migrants seeking, in the consumption of this product, an approximation to its territory.

According to Brazilian legislation, milk for cheesemaking must be pasteurised or thermal-treated (Ministério da Agricultura, do Abastecimento e da Reforma Agrária 1996). Pasteurisation eliminates pathogens, but also reduces the indigenous milk biota, causing changes to the sensory characteristics of cheeses (Dolci *et al.* 2008; Nikolic *et al.* 2008). In fact, the biodiversity of bacteria involved in cheese production can be considered a fundamental factor in maintaining the typical features of traditional cheese products. As a consequence, there is a demand for the development of autochthonous cultures specifically for traditional cheeses that allow standardisation of their quality and safety without changing the properties of the product (Dolci *et al.* 2008).

The aim of this research was to characterise *Lactobacillus* strains isolated from artisanal coalho cheese, in order to select indigenous acid lactic bacteria (LAB), with technological potential for the development of an adequate lactic culture that allows coalho cheesemaking from pasteurised milk. This research was a preliminary study towards defining a specific culture for coalho cheese. Next, the use of the selected culture for coalho cheesemaking will be adjusted to determine the technological steps to achieve coalho cheese made with pasteurised milk.

MATERIALS AND METHODS

Bacterial strains

Sixteen wild *Lactobacillus* strains from Embrapa Agroindústria Tropical Collection were selected for this work. *Lactobacillus* strains were

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previously isolated from raw bovine milk (two samples), curd (two samples) of different artisanal coalho cheesemaking and artisanal Brazilian coalho cheeses (14 samples) made with raw bovine milk, as described by Santos *et al.* (2015). After isolation, rod-shaped, Gram-positive and catalase-negative bacteria were submitted to the following tests: growth in sterile, reconstituted, commercial dry skim milk (RSM) at 10 g/100 mL, Molico, at 15 °C for 5 days and 45 °C for 2 days (Cogan *et al.* 1997); CO_2 production from glucose (Cogan *et al.* 1997); and ability to produce ammonia from arginine (Harrigan 1998). Presumptive lactobacilli were pre-identified by API50CHL systems (BioMérieux®, Marcy-l'Etoile, France). *Lactobacillus acidophilus* (ATCC4256), *Lactobacillus fermentum* (ATCC9338) and *Lactobacillus plantarum* (ATCC8914) were used as controls for the identification by API system. All micro-organisms were stored frozen at −80 °C in MRS broth (Biokar, Beauvais, France) added with glycerol 15 mL/100 mL.

To study antibacterial activity of these selected *Lactobacillus* strains, *Salmonella* sp. (OMS-Ca), *Staphylococcus aureus* (strain 76) and *Escherichia coli* (strain V517) (INALIN Collection) were grown in tryptone soy (TS) broth (Britania, Buenos Aires, Argentina) or nutrient agar (Britania), while *Listeria monocytogenes* (ATCC15313) was grown in brain heart infusion (BHI) broth (Britania) at 37 °C.

Enterococcus faecium strains EFM 568, EFM 577 and EFM 664 (INALIN Collection), used as positive controls in the assay of amine biogenic production, were cultivated in TS broth at 37 °C, while micro-organisms for bacterial interactions assays (*Streptococcus thermophilus* strains 32, 42, 43 and 179, INALIN Collection) were cultivated in Elliker broth (Biokar) at 43 °C.

Identification of *Lactobacillus* strains

Total DNA of isolates was obtained from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. Purified DNA samples were stored at −20 °C until use. The identity of isolates was analysed by amplifying, sequencing and comparing a 1500-bp fragment within their 16S rRNA gene (Edwards *et al.* 1989). All PCRs were performed using 1 μL of appropriate diluted DNA as template, 5.0 U Taq DNA polymerase (Sigma-Aldrich), 10 mM dNTPs (Sigma-Aldrich) and 10 μM each primer (Sigma-Genosys, The Woodlands, TX, USA) in a final volume of 50 μL. Amplifications were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at 94 °C, 36 cycles of 1 min at 94 °C, 2 min at 51 °C and 2 min at 72 °C, and a final step of 7 min at 72 °C. The PCR products were separated on agarose gels (0.8 g/100 mL) in Tris–borate–EDTA (TBE) buffer, stained with GelRed (Biotium Inc., Hayward, CA, USA) and visualised under UV light (Sambrook and Russell 2001). Amplicons were purified with QIAquick® Gel Extraction Kit (Quiagen GMBH, Hilden, Germany), and their nucleotide sequences were determined by primer extension at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity of isolates was checked by nucleotide BLAST of the NCBI database (www.ncbi.nlm.nhi.gov/blast).

RAPD analysis

Random amplification of polymorphic DNA (RAPD-PCR) was applied to the wild lactobacillus strains, to determine their genetic diversity. Oligonucleotide primers M13 (5′-GAGGGTGGCGGTTCT-3′; Huey and Hall 1989; Stendid *et al.* 1994) and 1254 (5′-CCGCAGCCAA-3′; Akopyanz *et al.* 1992) were used in separate amplification assays. PCRs were carried out in a volume of 25 μL containing 2.5 μL of 10× buffer (Sigma-Aldrich), 10 mM of dNTPs, primer M13 (1.0 μM) or primer 1254 (0.8 μM), 2.5 U of Taq DNA polymerase (Sigma-Aldrich) and 1.0 μL of appropriate diluted DNA template. PCR programs differed according to the primer used; for primer M13: one initial cycle of 3 min at 94 °C, followed by 35 cycles of 2 min at 94 °C, 20 s at 45 °C and 2 min at 72 °C; for primer 1254: four initial cycles of 5 min at 94 °C, 5 min at 36 °C and 5 min at 72 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. Both programs (M13 and 1254) ended with an extension step at 72 °C for 7 min. Amplified DNA fragments were resolved by electrophoresis on agarose gels (1.8 g/100 mL) precasted with GelRed™ (Biotium Inc.) as nucleic acid binding dye, in 1× TBE, and photographed under UV illumination; 1-kbp DNA ladder (GE Healthcare Life Sciences, Bucks, UK) was used as molecular weight marker.

Determination of acidifying character and proteolytic activities

The fast or slow acidifying character of the strains was determined as described by Briggiler Marcó *et al.* (2007). Strain cultures were inoculated (3 mL/100 mL) in RSM supplemented with or without glucose (1 g/100 mL) or with casein hydrolysate (0.25 g/100 mL), and milk supplemented with both glucose (1 g/100 mL) and casein hydrolysate (0.25 g/100 mL), followed by the assessment of pH after 24 h of incubation at 37 °C.

For those strains capable of growing in all conditions (RSM; RSM and glucose; RSM and casein hydrolysate; and RSM with glucose and casein hydrolysate) after two consecutive inoculations, milk acidification kinetics, and proteolytic (o-phthaldialdehyde spectrophotometric assay, OPA test, Church *et al.* 1983) and acidifying activities were determined by inoculation of the strains (3 mL/100 mL) in RSM followed by 24 h of incubation at 37 °C. The pH values were measured with a model SA 720 pH meter (Orion, Beverly, MA, USA) within 24 h. Proteolytic activity was

expressed as the difference in absorbance at 340 nm (A_{340}) between strain cultures and a control of milk. The developed acidity, measured by titration with N/9 NaOH to pH 8.4, was expressed as percentage of lactic acid. Slime production was visually determined by the increase of milk viscosity after incubation of each lactobacillus at 37 °C for 24 h in milk.

Salt resistance

Salt resistance was studied in MRS broth containing 1 or 2 g/100 mL of NaCl or *KCI* (Reddy and Marth 1993). Overnight culture of each *Lactobacillus* was inoculated (2 mL/100 mL) in each broth at 37 °C/24 h. After that, absorbance at 560 nm was measured and compared with a control without salt addition. The results were expressed as the percentage of growth in the presence of NaCl or KCl compared with the control.

Bacteriophage sensitivity

Lactobacillus strains were tested for their sensitivity to bacteriophages using a turbidity test, as described by Svensson and Christiansson (1991). The collection of three lytic phages was as follows: J-1 (sensitive strain *Lactobacillus casei* ATCC 27139), ATCC 8014-B1 and ATCC 8014-B2 (sensitive strain *Lb. plantarum* ATCC 8014), and four phages previously isolated in INLAIN from faulty batches of fermented milks in dairy industry: MLC-A (Capra *et al.* 2006), MLC-A2 and MLC-A8 (Capra *et al.* 2009) and C_L1 (Capra *et al.* 2010) (sensitive strain *Lactobacillus paracasei* A).

Biogenic amine production

The potential to produce biogenic amines tyramine, histamine, putrescine and cadaverine was assayed as described by Bover-Cid and Holzapfel (1999). Precursor amino acids, L-tyrosine disodium salt hydrate (Sigma-Aldrich), L-histidine monohydrochloride monohydrate (Sigma-Aldrich), L-ornithine monohydrochloride (Sigma-Aldrich) and L-lysine (Sigma-Aldrich) of each 1 g/100 mL were added to the culture medium containing 0.006 g/100 mL bromocresol purple. Each lactobacillus culture was streaked in triplicate on the medium plates with each precursor amino acid. *E. faecium* strains were used as positive controls. Petri dishes were incubated at 37 °C for 4 days under microaerophilic conditions. Positive reactions were recorded when a purple colour appeared on the plates or tyrosine precipitates disappeared around the colonies.

Antibacterial activity and nature of the pathogen inhibitory compound(s)

The well-diffusion agar assay was used as described by Vinderola *et al.* (2002). Cell-free supernatants (CFSs) of the sixteen wild lactobacillus strains were obtained by centrifugation of overnight cultures (7000 *g*, 15 min, 5 °C) and further sterilisation by filtration through a 0.22-μm pore filter (Millipore, São Paulo, Brazil). For the preparation of plates containing pathogens, nutrient agar (*Salmonella* sp. OMS-Ca, *S. aureus* 76 and *E. coli* V517) or BHI agar (*L. monocytogenes* ATCC15313) was melted and tempered at 45 °C, then vigorously mixed with an overnight culture of a pathogen (OD_{560} of 0.8) and poured onto a Petri dish. Wells of 10 mm in diameter were made in the agar layer, and 180 μL of CFS from each *Lactobacillus* strain was placed in a well. Plates were overnight incubated at 37 °C, and the diameters of the inhibition halos were recorded.

To elucidate the nature of inhibitor agent, CFSs of each *Lactobacillus* strain were subjected to the following treatments: heating at 121 °C for 15 min, neutralisation (pH 7.0) with 1 M NaOH and 1-hr incubation at 37 °C in the presence of 200 μg/mL proteinase K (Invitrogen Life Technologies, Carlsbad, CA, USA) or 200 μg/mL pepsin (Merck, Darmstadt, Germany). Treated CFSs were sterilised by filtration and assayed for remaining activity by the well-diffusion agar assay (Guglielmotti *et al.* 2007). CFSs without any treatment were used as controls.

Bacterial interactions

Well-diffusion agar assay (Vinderola *et al.* 2002) was also used to investigate interactions between wild Lactobacilli strains and between wild Lactobacilli and *Str. thermophilus* strains (strains 32, 42, 43 and 179). Streptococci CFSs was obtained as described in Section 2.8 for lactobacilli. The nature of the inhibitory compound(s) was evaluated as previously described (section 'Antibacterial activity and nature of the pathogen inhibitory compound(s)').

Cheese manufacture

Four Lactobacilli strains were selected from the results of the *in vitro* tests and assayed as adjunct cultures in cheesemaking experiments at plant pilot scale. Lactic cultures were designed, with two of these selected lactobacilli (coded as Lb) and two *Str. thermophilus* strains (coded as St), according to the following scheme: (i) culture 1: Lb BRM 032754 + Lb BRM 029692 + St 175 + St 42, (ii) culture 2: Lb BRM 029691 + Lb BRM 029693 + St 175 + St 42, (iii) culture 3: Lb BRM 029692 + Lb BRM 029693 + St 175 + St 42 and (iv) culture 4: Lb BRM 032754 + Lb BRM 029693 + St 175 + St 42. Each strain was cultivated separately in RSM until 10^9 colony-forming unit (CFU)/mL and mixed in equal proportions to compose the cultures as described above. Coalho cheesemaking experiments consisted of four different trials, one for each culture combination, according to the scheme of Figure 1. Raw milk was obtained from a nearby dairy factory (Milkaut S.A., Franck, Santa Fe, Argentina). It was batch-pasteurised (30 L) at 65 °C for 30 min and cooled to 35 °C. Next, calcium chloride (Merk), lactic culture (final concentration of 10^6 CFU/mL) and liquid rennet (Chy-max, Chr. Hansen, Buenos Aires, Argentina) were added to a final concentration of 0.02 g/100 mL, 2 mL/100 mL and 0.05 mL/100 mL,

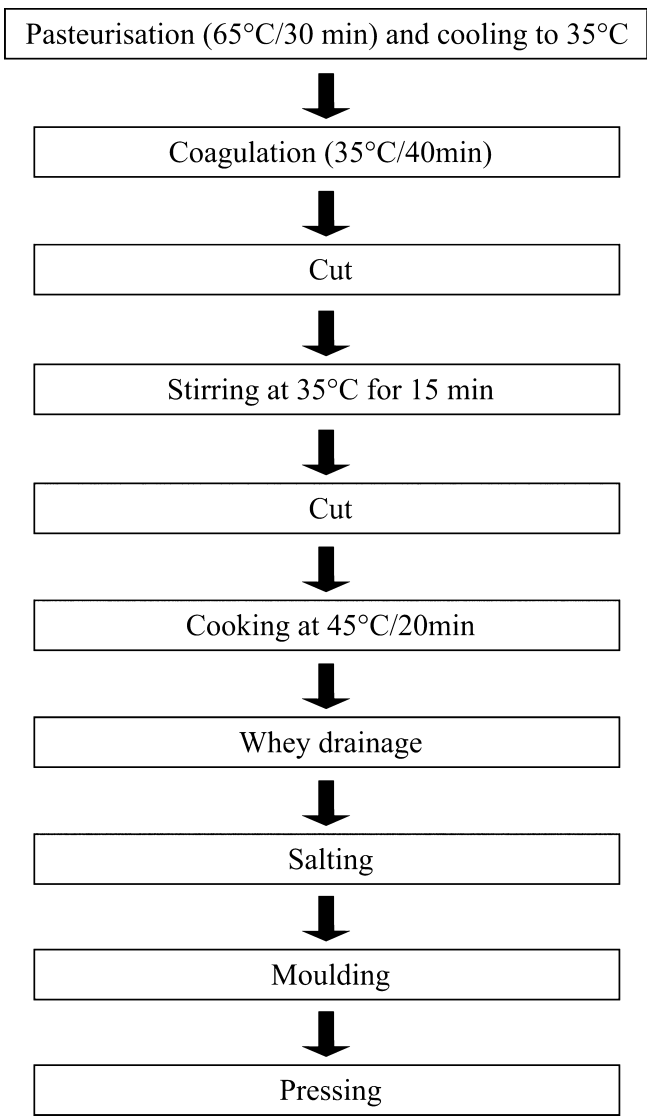

Figure 1 Scheme of coalho cheesemaking.

respectively, in the milk and maintained at 35 °C for 40 min to achieve coagulation. Curd was cut into cubes of approximately 1.0 cm^3. The curd grains were then gently stirred for 15 min at 35 °C, and the curd was cut again to adjust the size of curd particles. Next, the temperature was increased to 45 °C and the curd was cooked for 20 min under stirring. After the whey was drained, curd was salted by addition of sodium chloride at 1 g/100 mL and put in the moulds. Cheeses were pressed for 30 min (0.4 kg/cm^2) and stored at 4 °C without package for 15 days. Physico-chemical and microbiological parameters were assessed. Fat content (IDF 1997) and moisture (IDF 1982) were quantified in the cheese samples stored for 7 days; pH was measured at time 1, 7 and 14 days (Bradley *et al.* 1993). Microflora (mesophilic micro-organisms, LAB, coliforms, yeast and moulds) was also determined as follows: samples (10 g) were aseptically taken from each cheese and homogenised for 3 min in a stomacher laboratory blender (PBI International, Milan, Italy) with sterile sodium citrate solution (2 g/100 mL). After decimal dilutions in sterile peptone water (0.1 g/100 mL), viable counts were determined in plate count agar (PCA, Britania) at 32 °C for 48 h in aerobic conditions; PCA was added with RSM (10 mL/100 mL) at 37 °C for 48 h under microaerophilic conditions, violet red bile agar (VRBA; Britania) at 32 °C for 24 h and chloramphenicol glucose agar (CGA; Biokar) at 32 °C for 7 days.

RESULTS AND DISCUSSION

Isolation, identification and genetic diversity of lactobacilli isolated from Brazilian artisanal Coalho cheeses

Most microbiological studies of coalho cheese have been focused on its contaminant microbiota and showed it has a low microbiological quality, often associated with the use of raw milk and inadequate cheese processing conditions (Machado *et al.* 2011). Therefore, to improve quality, a screening of LAB from coalho cheese, aiming to formulate a suitable culture to be inoculated in pasteurised milk for its manufacture, was proposed. Micro-organisms were isolated from raw bovine milk, curd and artisanal coalho cheeses made with raw milk. Cheeses were from Jaguaribe (10 samples), Maranguape (one sample), Tauá (two sample) and Quixadá (one sample) towns of Ceará state, while raw milk and curd were from Jaguaribe producers. A total of 217 isolates were preliminarily classified as rod-shaped bacteria. Gram-positive, catalase-negative and acid producer micro-organisms were considered as presumptive lactobacilli. Then, their ability to grow in skim milk at 15 and 45 °C and to produce CO_2 from glucose and ammonia from arginine was examined. During the tests, the number of presumptive lactobacilli diminished because some of them did not support the successive cultivations. Based on these results, a total of 16 *Lactobacillus* strains were selected. Most of them grown at 45 °C but not at 15 °C did not produce CO_2 from glucose and none was able to produce ammonium from arginine. Two of them (BRM 032532 and BRM 032753) were able to grow at 15 °C but not at 45 °C, and the other two (BRM 032754 and BRM 032755) formed gas from glucose. An API50CHL system was used to investigate their ability to ferment sugars and to make a preliminary identification. Identification based on API results failed for ten strains. Strain BRM 032532 was found to be *Lactobacillus brevis*, strains BRM 032753 and BRM 032529 were classified as *Lb. plantarum* and strains BRM 032528, BRM 029693 and BRM 029728 were classified as *Lb. paracasei*. All 16 strains were subjected to 16S rDNA sequencing. According to this, 13 strains were classified as

Lactobacillus rhamnosus, two as *Lb. fermentum* and one as *Lb. plantarum* (Table 1). Our results agree with Santos *et al.* (2015) who found 28 different *Lb. rhamnosus* strains, five *Lb. plantarum* strains and one *Lb. fermentum* strain from a total of 34 strains isolated from artisanal coalho cheeses. Moreover, *Lactobacillus* species have also been found in other sorts of raw milk cheeses (Briggiler Marcó *et al.* 2007; Bove *et al.* 2011; Singh and Singh 2014).

To determine the genetic diversity of the lactobacilli, RAPD-PCR was applied using primers M13 and 1254. Bionumeric analysis of the DNA combined profiles showed six patterns at 81% of similarity degree (Figure 2). According to that, the two *Lb. fermentum* strains belong to the same cluster, while it was possible to define two clusters for *Lb. rhamnosus* strains and two independent strains. The RAPD profile of *Lb. plantarum* strain was also independent from the others. The knowledge about taxonomy and phylogeny obtained from molecular data were used in combination with technologic characterisation to further selection for coalho cheese adjunct cultures.

Technological characterisation

The strains were classified as slow or fast according to the final pH reached by their RSM cultures after 24 h at 37 °C. In this work, we did not find fast strains. Ten *Lb. rhamnosus* were able to grow in all evaluated conditions (RSM; RSM and glucose; RSM and casein hydrolysate; and RSM, glucose and casein hydrolysate) and were classified as slow strains with final pH values in milk ranging between 4.52 ± 0.08 and 5.13 ± 0.16 after 24 h of incubation. *Lb. fermentum* strains, *Lb. plantarum* and three strains of *Lb. rhamnosus* (BRM 032752, BRM 032753 and BRM 032530) only grew in RSM supplemented with casein hydrolysate, glucose or both. Georgieva *et al.* (2009) studied eight *Lb. plantarum* strains isolated from artisanal Bulgarian white cheeses and characterised them as slow variants. Briggiler Marcó *et al.* (2007) found 70% of slow strains, including species of *Lb. rhamnosus*, *Lb. plantarum* and *Lb. fermentum*, from a total of 23 *Lactobacillus* strains isolated from soft and semihard Argentinean cheeses. In our study, only the *Lb. rhamnosus* strains able to grow in RSM were assayed for milk acidification kinetics, acidifying and proteolytic activities, but four of them did not support the successive cultivations required until the end of the test. The remaining strains (BRM 032527, BRM 032750, BRM 032532, BRM 029691, BRM 029693 and BRM 029728) caused a slow decrease in milk pH, as shown by their milk acidification curves, reaching pH values ranging from 4.63 ± 0.24 to 5.13 ± 0.11 after 24 h (Figure 3). Acidifying activities of the strains were low, ranging between $0.49 \pm 0.01\%$ (strain BRM 032527) and $0.63 \pm 0.02\%$ of lactic acid (strain BRM 029691) produced. Proteolytic activities were also poor, varying from 0.016 ± 0.010 (strain BRM 029728) to 0.120 ± 0.006 (strain BRM 032750). Briggiler Marcó *et al.* (2007), Georgieva *et al.* (2009) and Nieto-Arribas *et al.* (2009) also found low acidifying activity and a variable proteolytic activity in species of *Lb. rhamnosus* and *Lb. plantarum* isolated from a variety of cheeses. These low activities are not considered a limitation for the use of these bacteria in coalho cheese manufacture as one of the desirable characteristics is a slight acidity in cheese. This allows the coalho cheese to be roasted without melting, maintaining the integrity of its form. The selection of strains with low acidifying activity will contribute to the development of a slightly acidic product. Moreover, coalho cheese does not undergo a ripening process, and the low proteolytic activity showed by these strains is expected, as they were isolated from the artisanal product.

Among the tested bacteria, the production of slime by the BRM 029693 strain, observed visually by the viscosity increase when this strain grew in milk, was remarkable. According to Nieto-Arribas *et al.* (2010) in dairy technology, the production of slime is considered to be a relevant feature for LAB cultures, because they act as texturisers and stabilisers, and smooth creamy products have considerable appeal for consumers. One of the characteristics of coalho cheese is this rubbery texture, and the presence of slime producer strains may possibly contribute to this.

In the presence of both NaCl and KCl at concentrations of 1 and 2 g/100 g, all strains showed growth above 60% (Table 1). The strains seemed to be adapted to both salts, once minor differences between the growth of the control and the growth of the media with salt were noticed. In fact, it was also observed that salts slightly improved the growth of some strains. This result is not surprising because during coalho cheese manufacture, salt is generally added directly to curd at levels of 1–2 g/100 g, thus explaining the isolation of these strains from the artisanal cheeses, where they are probably adapted to the environmental conditions. Tolerance to salt was also reported by other authors. Georgieva *et al.* (2009) observed significant growth of eight *Lb. plantarum* strains in the presence of 3% NaCl and a good adaptation of the same strains when growth was promoted at 6% NaCl. Nikolic *et al.* (2008) evaluated 48 *Lactobacillus* sp. isolated from Bukulijac cheese to NaCl at 4, 6.5 and 8% and verified that all of them were resistant at 4%, 36 strains at 6.5% and none at 8% NaCl concentrations. Briggiler Marcó *et al.* (2007) found resistance to NaCl and KCl when assaying strains of *Lb. rhamnosus* and *Lb. plantarum* in media supplemented at 1, 2 and 3% of these salts.

Wild *Lb. rhamnosus* and *Lb. plantarum* strains were resistant to the seven bacteriophages tested (J-1, ATCC 8014-B1, ATCC 8014-B2, MLC-A, MLC-A2, MLC-A8 and C_L1), because they were able to exhibit a normal growth during three consecutive subcultures in phage presence. Phage resistance is an important characteristic when screening for new start or adjunct micro-organisms, because the use of phage-resistant cultures is one of the strategies to

Table 1 Classification of *Lactobacillus* strains based on 16SrDNA sequencing and technological characterisation

		Growth (%) in the presence of salt (%, w/v)				*Inhibition of*[a]			
Bacterial species	*Strain*	*NaCl 1%*	*NaCl 2%*	*KCl 1%*	*KCl 2%*	*Listeria monocytogenes* ATTC 15313	*Escherichia coli* V517	*Staphylococcus aureus* 76	*Salmonella* sp. OMS-Ca
Lactobacillus rhamnosus	BRM 032527	100.4 ± 0.9	90.4 ± 3.9	103.3 ± 0.3	95.5 ± 3.4	0.60 ± 0.02	0.51 ± 0.03	0.61 ± 0.03	0.40 ± 0.07
	BRM 032528	nd	nd	nd	nd	nd	nd	nd	nd
	BRM 032750	99.4 ± 1.9	108.1 ± 0.7	100.7 ± 2.9	90.9 ± 0.2	0.62 ± 0.01	0.42 ± 0.01	0.61 ± 0.01	0.60 ± 0.02
	BRM 032751	nd	nd	nd	nd	nd	nd	nd	nd
	BRM 032532	101.7 ± 5.2	89.5 ± 3.8	104.5 ± 2.3	83.9 ± 7.6	0.71 ± 0.03	0.32 ± 0.04	0.62 ± 0.01	0.44 ± 0.01
	BRM 032752	nd	nd	nd	nd	nd	nd	nd	nd
	BRM 032753	nd	nd	nd	nd	nd	nd	nd	nd
	BRM 032529	91.1 ± 5.8	98.5 ± 1.0	90.6 ± 3.3	103.1 ± 1.1	nd	nd	nd	nd
	BRM 032530	nd	nd	nd	nd	nd	nd	nd	nd
	BRM 032531	nd	nd	nd	nd	nd	nd	nd	nd
	BRM 029691	99.9 ± 5.5	82.5 ± 5.0	98.8 ± 5.1	98.3 ± 1.8	98.3 ± 1.8	0.32 ± 0.01	0.52 ± 0.04	0.62 ± 0.04
	BRM 029693	102.7 ± 0.6	108.0 ± 0.6	101.7 ± 0.8	86.8 ± 1.6	0.62 ± 0.01	0.33 ± 0.06	0.71 ± 0.03	0.54 ± 0.06
	BRM 029728	87.2 ± 3.5	97.5 ± 2.7	98.1 ± 5.3	103.0 ± 5.0	0.62 ± 0.02	0.41 ± 0.06	0.73 ± 0.03	0.52 ± 0.01
Lactobacillus plantarum	BRM 029692	104.2 ± 1.7	106.7 ± 0.6	100.0 ± 5.8	101.8 ± 1.2	0.61 ± 0.04	0.52 ± 0.01	0.86 ± 0.02	0.65 ± 0.04
Lactobacillus fermentum	BRM 032755	69.4 ± 4.9	101.0 ± 3.4	112.7 ± 4.7	107.3 ± 5.4	g	g	g	g
	BRM 032754	107.0 ± 2.1	105.7 ± 2.3	87.1 ± 1.9	118.2 ± 5.2	g	g	g	g

nd, not determined; g, growth around the halo.

[a]Inhibition halo diameter (cm) – well diameter (1 cm).

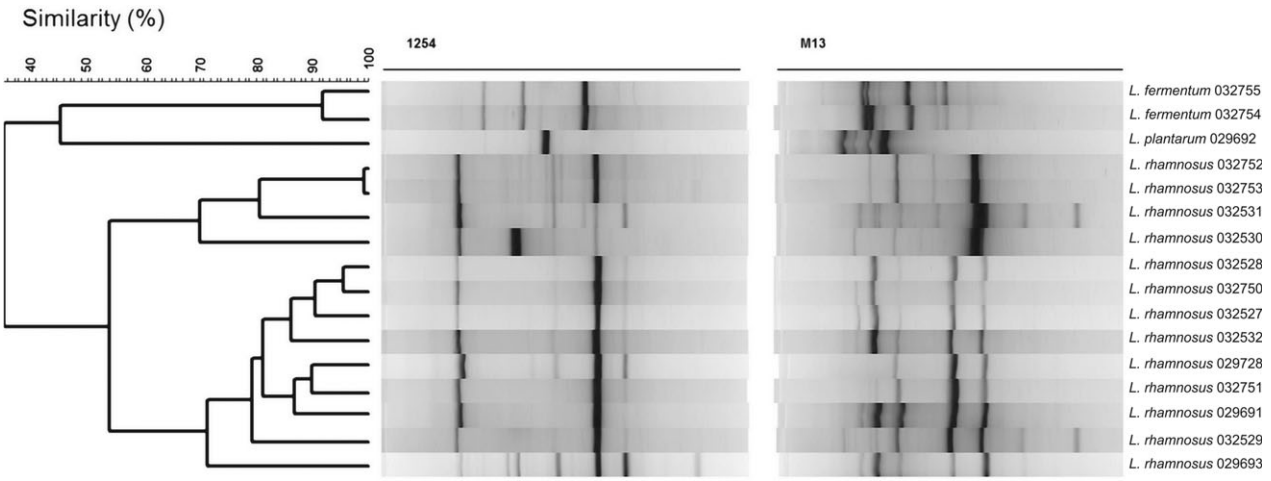

Figure 2 RAPD-PCR profiles obtained from the 16 wild *Lactobacillus* strains using primers 1254 and M13, and corresponding dendrogram derived from the unweighted pair group average linkage of Pearson correlation coefficients (expressed as a percentage value). Names of the strains are indicated on the right hand side.

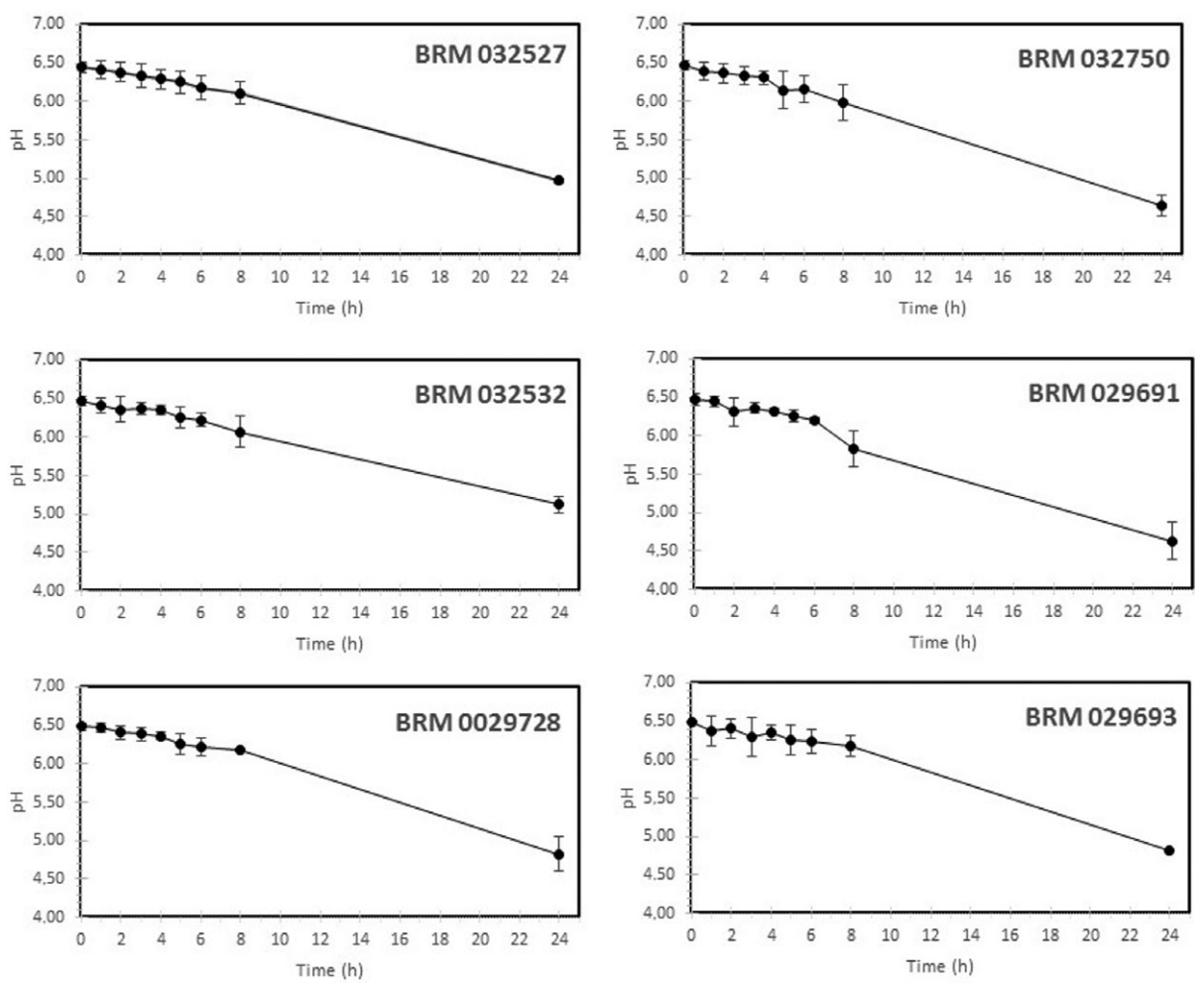

Figure 3 Milk acidification kinetics (37°C, 24 h) of *Lactobacillus rhamnosus* BRM 032527, BRM 032750, BRM 032532, BRM 029691, BRM 0029728 and BRM 029693.

minimise problems caused by phage attacks in dairy plants (Briggiler Marcó *et al.* 2011).

None of the tested *Lactobacillus* strains were able to decarboxylate L-tyrosine, L-histidine, L-ornithine and L-lysine. Biogenic amines have toxicological effects and its biogeneration is acknowledged as an undesirable feature of food-grade bacteria (Bover-Cid and Holzapfel 1999; Nieto-Arribas *et al.* 2009). Santos *et al.* (2015) looked for the presence of genes for histidine decarboxylase (*hdc1* and *hdc*2), ornithine decarboxylase (*odc*) and tyrosine decarboxylase (*tdc*) in five *Lb. rhamnosus* strains and three *Lb. plantarum* strains isolated from coalho cheese and found that four *Lb. rhamnosus* strains were positive for *hdc1* gene. Songisepp *et al.* (2012) evaluated safety of the probiotic *Lb. plantarum* strain Tensia (DSM 21380) in semihard Edam-type cheese and verified the absence of harmful biogenic amines, such as histamine or cadaverine, while the amount of tyramine produced in the cheese environment during ripening was below the clinically significant content. Nieto-Arribas *et al.* (2010) did not find any decarboxylase activity when evaluating 23 *Leuconostoc* isolates from artisanal manchego cheese. The same author also examined biogenic amine production of 30 *Lactobacillus* strains and verified that only one strain of *Lb. paracasei* subsp. *paracasei* was able to decarboxylate L-tyrosine (Nieto-Arribas *et al.* 2009). The use of strains with low *in vitro* decarboxylase activity can be strategic to reduce biogenic amine accumulation in cheeses, though Nieto-Arribas *et al.* (2010) highlight that negative results for biogenic amine production in the laboratory do not necessarily imply a similar behaviour in cheese, as some authors reported that factors such as pH, NaCl concentration and availability of substrate can influence the build-up of biogenic amines.

Possible interactions between *Lactobacillus* strains were investigated by the agar diffusion assay. It was verified that none of them inhibited the growth of the other. The influence of lactobacilli on the growth of wild *St. thermophilus* strains from INLAIN collection was also tested. Inhibition of *St. thermophilus* was first observed when *Lactobacillus* CFSs were inoculated on the well. The pH values of CFSs were around 4.0, and to verify whether this low pH was responsible for the observed inhibition, CFSs were neutralised to pH 7.0 and the well-diffusion assay was repeated. After CFS neutralisation, the four *St. thermophilus* strains were able to grow. In turn, streptococci CFSs did not inhibit lactobacillus growth. As previously acknowledged, the inhibitory effects of LAB species are variable. Santos *et al.* (2015) studied the effect of *Lb. plantarum* and *Lb. rhamnosus* strains on the growth of commercial cultures (*Lactococcus lactis* and *St. thermophilus*) used in dairy processing, and verified that all lactobacilli CFSs slightly inhibited the growth of the starter cultures. Similarly, Lavilla-Lerma *et al.* (2013) studied 80 lactobacillus strains belonging to *Lb. plantarum* and *Lb. paracasei* species and found that 12 *Lb. plantarum* and eight *Lb. paracasei* strains were producers of bacteriocin-like inhibitors against indicator strains *Lb. plantarum* CECT748 and *Lb. paracasei* CECT4022. Vinderola *et al.* (2002) isolated 17 *Lb. rhamnosus* strains and two *Lactobacillus gasseri* strains from the faeces of new-born infants and showed that none of the strains inhibited the growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains, but they were inhibitory towards *Lac. lactis* and *St. thermophilus* strains. They also observed no effects of the CFSs of *Lb. delbrueckii* subsp. *bulgaricus, Lac. lactis* and *St. thermophilus* strains on intestinal *Lactobacillus* growth.

In our work, nine strains (BRM 032527, BRM 032750, BRM 032754, BRM 032532, BRM 029691, BRM 032755, BRM 029692, BRM 029693 and BRM 029728) were also investigated for antimicrobial activity against *L. monocytogenes*, *E. coli*, *Staph. aureus* and *Salmonella* sp. Except *Lb. fermentum* strains (BRM 032754 and BRM 032755), all micro-organisms showed inhibition halos around the well (Table 1). All diameters of halos produced were <10 mm. Furthermore, there was not noticed any difference in inhibitory effect on gram positive (*Lb. monocytogenes* and *Staph. aureus*) and *Salmonella* sp., as the halo diameters produced by these micro-organisms were very similar. In contrast, the halo produced against *E. coli* was smaller than the halo against the Gram-positive bacteria. Additionally, the lactobacillus CFSs from strains BRM 029691, BRM 029692 and BRM 029693 were neutralised, autoclaved and incubated with proteinase K and pepsin, with the aim of elucidating the nature of the inhibitory agent. Results showed that the antibacterial effect disappeared when the CFSs were neutralised. After treatment with pepsin, the halo intensity produced by CFSs of the two *Lb. rhamnosus* strains (BRM 029691 and BRM 029693) was reduced against all pathogens, except for *E. coli*. CFSs of *Lb. plantarum* BRM 029692 treated with pepsin produced a weak halo only against *Salmonella* sp. Although the main antibacterial effect was caused by acidity pH of supernatants, it is possible that some substance of proteinaceous nature may also have inhibited the pathogenic bacteria, but further research is necessary to clarify this. *Lactobacillus* antibacterial activity against pathogenic bacteria was also observed by other researches (Guglielmotti *et al.* 2007; Nikolic *et al.* 2008; Vinderola *et al.* 2008; Lavilla-Lerma *et al.* 2013; Santos *et al.* 2015).

Selection of strains to coalho cheese manufacture

Taking into account the results previously detailed, four strains were chosen to be evaluated for coalho cheese manufacture: BRM 032754, BRM 029691, BRM 029692 and BRM 029693. *Lb. fermentum* BRM 032754 and *Lb. plantarum* BRM 029692 were the less acidifying strains, while *Lb. rhamnosus* BRM 029691 was the most acidifying strain from the isolates evaluated. *Lb. rhamnosus* BRM

Table 2 Coalho cheese characterisation

					pH after			*Counts (log CFU/g)*											
								Coliforms			*Mesophiles*			*LAB*			*Yeast and mould*		
					Time (days)														
Culture	*Strains*	*Characteristics*	*Fat (%)*	*Moisture (%)*	*1*	*7*	*14*	*1*	*7*	*14*	*1*	*7*	*14*	*1*	*7*	*14*	*1*	*7*	*14*
1	St 42, St 175, BRM 032754 and BRM 029692	Both BRM 032754 and BRM 029692 are low acidifying and fastidious	32	46.5	5.2	5.5	5.3	2.7	2.2	2.8	8.9	9	8.6	9.1	9.2	9.2	<1	<1	<1
2	St 42, St 175, BRM 029691 and BRM 029693	Both BRM 029691 and BRM 029693 belong to the same species, they are the most acidifying among the studied strains and BRM 029693 is a slime producer	35	45.0	5.1	5.4	5.1	2.1	2.7	2.8	9.2	9.1	9.3	9.4	9.3	9.4	<1	<1	<1
3	St 42, St 175, BRM 029692 and BRM 029693	*Lactobacillus* are from different species (plantarum and rhamnosus), and BRM 029693 is a slime producer	31	46.5	5.3	5.5	5.2	2.6	2.3	2	9.1	8.8	9	9.2	9.2	9.3	<1	<1	<1
4	St 42, St 175, BRM 032754 and BRM 029693	*Lactobacillus* are from different species (fermentum and rhamnosus), and BRM 029693 is slime producer	33	45.0	5.3	5.5	5.2	2.4	3.0	2.6	9.0	9.2	9.1	9.2	9.2	9.2	<1	<1	<1

St, *Streptococcus thermophilus*.

029693 was selected mainly by the slime production, which may contribute to some sensorial characteristics of coalho cheese. Furthermore, according to the results of the molecular analyses, the selected strains showed genetic diversity. To find the best culture composition, selected strains were grouped as shown in Table 2. Two *St. thermophilus* strains (St 42 and St 175) from INLAIN collection were used in all formulations, although the *Lactobacillus* strains varied in each culture. All micro-organisms were added in the same proportions.

Cheese manufacture

Four different coalho cheeses were made, one for each selected culture. Fat and moisture content were around 33 g/100 g and 46 g/100 g, respectively (Table 2). The pH was determined at times 1, 7 and 14 days. The cheeses exhibited pH values between 5.1 and 5.5. During storage time, the pH of all the cheeses showed a slight increase on day 7, followed by a decrease to values close to the initial pH on day 14 (Table 2). There is no legislation for the pH range in coalho cheese, but this product is usually consumed roasted, with the preservation of its initial form. Thus, to avoid melting, pH of coalho cheese should be around 6.0, because a higher pH implies higher calcium content in the cheese, maintaining it firmer during cooking.

After 24 h (day 1) of cheesemaking, the lactic cultures reached numbers of the order of 9 logs and they remained viable at the same level in all cheeses during storage for 14 days (Table 2). Mesophilic counts were also in the order of 8–9 logs during the analyses, as expected for fermented products like cheese, while coliform counts were around the level of 2 logs. Other parameters of the conditions of food processing were yeasts and mould counts, which were low (<1 log order) for all the cheeses during storage.

CONCLUSION

It was demonstrated that it was possible to manufacture coalho cheese by adding the selected *Lactobacillus* strains as adjunct culture. Nevertheless, the optimal balance of these strains in order to avoid excessive acidification is still being investigated, as pH is a very relevant technological parameter in this kind of cheese.

ACKNOWLEDGEMENTS

We are grateful to A. Binetti and G. George for technical assistance. L. Bruno was the recipient of a postdoctoral international fellowship awarded by Embrapa – Empresa Brasileira de Pesquisa Agropecuária. This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; Project PIP No 11220080101206; Argentina); the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Project PICT 2010-0138; Argentina) and EMBRAPA (Embrapa; Project 03.12.01.006.00.00, Brazil).

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