Characterization of technological and probiotic properties of indigenous *Lactobacillus* spp. from south Brazil

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Abstract

In this study, we isolated *Lactobacillus* spp. from bovine raw milk and artisanal cheese from southern Brazil, and evaluated their technological and probiotic potential to select new isolates for producing healthy fermented dairy foods with differentiated tastes and flavours. We obtained 48 new lactobacilli isolates, which were isolated from raw milk (38) and cheese (10). These bacterial isolates were closely related with ten species: *Lactobacillus paracasei* (50% of the isolates), *L. parabuchneri* (15%), *L. pentosus* (13%), *L. zae* (4%), *L. plantarum* (4%), *L. otakiensis* (4%), *L. casei* (4%), *L. harbinensis* (2%), *L. diolivorans* (2%), and *L. rhamnosus* (2%). Isolates CH112 and CH131 showed the greatest acidification potential, reducing the pH of milk to below 5.3 after incubation for 6 h at 32 °C. Considering proteolytic activity and diacetyl production, isolates ML88a, ML04, and ML12 showed the most promising results. Isolate ML12 showed 100% survival rate when inoculated in gastric juice at pH 2.5. The evaluation of antibacterial activity of the lactobacilli showed that the pathogens *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis*, and *Salmonella Typhimurium* were strongly inhibited by the pure lactobacilli cultures. Five *Lactobacillus* isolates (ML01, ML04, ML12, ML88, and CH139) showed both technological and probiotic characteristics. Principal Component Analysis (PCA) was used to investigate correlations among technological and probiotic characteristics, and identified new promising lactobacilli isolates for exploring their characteristics. This study reveals the importance of selecting new microorganisms with potential applicability in the food industry for developing functional foods with differentiated aromas and flavours.

Keyword Brazil. Fermented dairy foods. Functional foods. Lactic acid bacteria. Lactobacilli isolates. Starter culture.

Introduction

Lactic acid bacteria (LAB) constitute one of the most important bacterial groups used for the processing of fermented dairy, meat, vegetable, and cereal products (Hurtado et al. 2012). LAB metabolize food matrix constituents, producing large amounts of compounds, such as organic acids, aldehydes, alcohols, esters, peptides, amino acids, and fatty acids (Oliszewski et al. 2013). These compounds play important roles in determining the taste, aroma, texture, and shelf life of fermented food products (Tamang et al. 2016), and consequently, LAB are often used as microbial inocula during food production processes. The studies of technological and safety properties of LAB from different origins (e.g., grape must, cheese, and human stomach) evidence that such microbial group has appropriate characteristics for many

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applications, such as wine production (Berbegal et al. 2016) and other fermented foods, including dairy (Delgado et al. 2015; Bujnakova and Strakova 2017). Isolation and selection of new wild-type LAB strains from raw food increase their potential for biotechnological application as starter culture for food fermentation, with the aim of producing a standardized and more stable product that preserves the food's traditional sensory characteristics (Benito et al. 2007).

In addition to their technological importance, many LAB species act as probiotics (Souza and Dias 2017). Probiotic bacteria as a non-pathogenic constituent of the natural intestinal flora play important roles preserving health and well-being of the human host (Forestier et al. 2001). Their ingestion confers numerous health benefits, such as stimulation and regulation of the immune system (Angmo et al. 2016) and control of pathogen overgrowth, thereby controlling or preventing infections and protecting the intestinal epithelium through a series of barriers and interference mechanisms (Forestier et al. 2001). LAB also alleviate lactose intolerance and may lower cholesterol levels. *Lactobacillus* spp. are Generally Recognized As Safe (GRAS), showing suitable use as starter cultures in the food industry, and their presence in the human gastrointestinal tract has beneficial effects on health. When evaluated for probiotic potential, these cultures must also be tested on their functional properties and safety attributes (Bujnakova and Strakova 2017). Technological and probiotic potential of LAB are strain specific, and selected *Lactobacillus* spp. strains have been successfully used as starter and probiotic inoculants in fermented dairy products (Forestier et al. 2001).

For many years, probiotic potential of selected LAB has been evaluated according to their resistance to gastric and intestinal juices, antimicrobial activity against pathogens, resistance to antibiotics, and antioxidant activity (Bernardeau et al. 2008; Burns et al. 2012; Tokatlj et al. 2015; Frau et al. 2016; Kumari et al. 2016; Bujnakova and Strakova 2017). The search for new probiotic strains is a crucial requirement for a successful development of the functional fermented foods market. Guidelines developed by the United Nations FAO/WHO include important recommendations for the development and evaluation of probiotic foods, such as: trustworthy identification of the bacterial species; *in vitro* before *in vivo* tests as a first step; and a guarantee that probiotic strains are safe and contamination free (Messaoudi et al. 2013).

Some studies have investigated dairy microbiota with the aim of selecting indigenous *Lactobacillus* spp. with the potential to be used at the industry of fermented dairy foods. Such foods must present sensorial characteristics similar to those observed in traditional products (Bernardeau et al. 2008; Burns et al. 2012; Owusu-Kwarteng et al. 2015; Tokatlj et al. 2015; Frau et al. 2016; Kumari et al. 2016). Thus, this work aimed to isolate new *Lactobacillus* spp.

from bovine raw milk and artisanal cheese from southern Brazil, and evaluate their technological and probiotic potential to select new isolates for producing healthy fermented dairy foods with differentiated tastes and flavours.

Materials and methods

Lactobacilli isolation

Ten samples of raw cow milk and ten samples of starter-free raw cow milk cheese were collected from local producers in the Taquari Valley region of southern Brazil (28°38'55" to 29°52'00" of South latitude and 51°31'04" to 52°33'11" of West longitude), and used for *Lactobacillus* spp. isolation. Bacterial isolation was performed on Man Rogosa & Sharpe (MRS) (Oxoid®, Basingstoke, England) and M17 agars (Oxoid®, Basingstoke, England) incubated at 32 °C for 72 h. Colonies with different morphologies were selected and purified on MRS agar. Bacterial isolates Gram-positive, rod-shaped, catalase-negative, and haemolysis-negative (Maragkoudakis et al. 2009) were selected and stored in glycerol 20% (v/v) at 20 °C for further investigation.

Identification of the bacterial isolates

Bacterial DNA was extracted according to Sambrook and Russel (2001). A fragment of approximately 1500 bp of *16S rRNA* gene was amplified via PCR using BacpaeF (5'-AGA GTTTGATCCTGGCTCAG-3') and Bac1542R (5'-AGA AAGGAGGTGATCCAGCC-3') primers (Stackebrandt and Liesack 1993). Amplification reactions contained 50 ng of DNA, 0.1 mM MgCl₂, 20 mM of each dNTP, 0.3 mM of each primer and 1 U of Taq DNA Polymerase (Invitrogen®, San Diego, California) in a final volume of 25 µL. Amplifications were performed as follows: an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, and one last cycle at 72 °C for 7 min for the final elongation step. PCR products were analyzed by electrophoresis in 1% agarose gels in Tris-Borate-EDTA (TBE) buffer with ethidium bromide and visualized by UV light.

PCR products were sequenced in a MegaBACE 1000 automatic sequencer using a DYEnamic™ ET Dye Terminator Cycle Sequencing Kit (GE Healthcare). Each DNA fragment was sequenced with the same forward and reverse primers used in PCR analysis (BacpaeF and 1542R). Sequence analyses were performed with BioEdit version 7.0.9.0 software (Hall 1999) to verify sequence quality and to check for possible chimeric origins. A fragment of approximately 1200 bp from the *16S rRNA* gene was compared with sequences from the EzTaxon-e Server (Chun et al. 2007). DNA sequences obtained in this work were

deposited in the GenBank database under accession numbers MF611797 to MF611844.

Technological properties of the Lactobacilli

Each lactobacilli was incubated in MRS broth at 32 °C until reaching a concentration of approximately 10^9 CFU/mL. Bacterial cells were centrifuged (5000×g, 15 min, 4 °C), washed twice in potassium phosphate buffer (10 mM, pH 7.0), and suspended in peptone water. These bacterial inocula were used to evaluate the technological characteristics listed below.

Acidification potential was evaluated by periodic measurements (0, 6, 9, and 24 h of incubation) of pH and acidity in three different broths: milk, cheese whey, and permeate. Each broth was inoculated with bacterial isolate and incubated at 7 °C and 32 °C. The pH value (Δ pH) and titratable acidity [% (w/v) lactic acid] were expressed as the variation between the initial time (0 h) and after incubation.

Proteolytic activity was evaluated on milk agar, which was prepared by adding 1% (w/v) skim milk powder to Plate Count Agar (Oxoid®, Basingstoke, England). One drop of 2 μ L of each bacterial suspension was inoculated in the milk agar plate and incubated for 72 h at 32 °C. The transparent halos formed around bacterial colonies were considered positive for proteolytic activity, and the halo sizes were measured (Beerens and Luquet 1990).

To evaluate diacetyl production, bacterial suspensions were individually inoculated (1% (v/v)) in 10 mL of sterilized whole milk and incubated at 32 °C for 24 h. Then, 1 mL of this bacterial suspension was added to 0.5 mL of α -naphthol solution [1% (w/v)] plus 0.5 mL of KOH [16% (w/v)] and incubated at 32 °C for 10 min. Production of diacetyl was indicated by the formation of a red ring on the tubes (King 1948). The results were classified as weak, medium, or strong, according to the intensity of the ring colour.

Resistance to gastric and intestinal juices

A simulated gastrointestinal tract (GIT) to test bacterial tolerance was performed according to Meira et al. (2012). Each lactobacilli isolate was inoculated on MRS broth and incubated at 32 °C for 48 h. Bacterial cells were centrifuged (5000×g, 15 min, 4 °C), washed twice with phosphate buffer (10 mM, pH 7.0), and suspended in NaCl solution [0.5% (w/v)]. An aliquot (0.2 mL) of each bacterial suspension was incubated at 37 °C in the presence of 1 mL of gastric and intestinal juices. Viable cell counts were performed on MRS agar in initial exposure to GIT juices (0 h) and after 3 h and 4 h for gastric and intestinal juices, respectively. Simulated gastric juice was prepared using 3.0 mg/mL pepsin from porcine gastric mucosa and 0.5% (w/v) NaCl. This solution was

acidified with HCl until pH reached values of 2.0, 2.5, and 3.0. Simulated intestinal juice was prepared with 1.0 mg/mL pancreatin and 0.5% (w/v) NaCl, and the pH was adjusted to 8.0, with or without 0.5% (w/v) of a 1:1 mixture of sodium cholate and sodium deoxycholate.

Antimicrobial activity

Inhibitory effects of the new lactobacilli isolates against bacterial pathogens *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC11778, *Salmonella Typhimurium* ATCC14028, *Salmonella enteritidis* ATCC13076, and *Listeria monocytogenes* ATCC19114 were evaluated by the disc-diffusion method. The lactobacilli cell preparations used for this assay were: pure bacterial growth (PG), pure supernatant (PS), neutralized supernatant (NS), heat-treated supernatant (TS), concentrated supernatant (CS), and intracellular extract (IE). Initially, each bacterial isolate was inoculated in MRS broth and incubated at 32 °C until reaching approximately 10^9 CFU/mL. An aliquot of this cell suspension (called PG) was removed and assayed for its antimicrobial activity. Then, the bacterial cells were centrifuged (5000×g, 15 min, 4 °C) and cell-free supernatant (called PS) was selected. An aliquot of this cell-free supernatant was neutralized (called NS). Another aliquot was heated at 95 °C for 5 min (called TS), and another aliquot was concentrated (2×) using a speed vacuum (called CS). Glass beads were added to the cell pellet and subjected (five times) to 30 s in an ice bath and 30 s in vortex agitation, and the resulting IE was also assayed for its antimicrobial activity.

Each pathogen was inoculated in Mueller–Hinton (MH) agar (Oxoid®, Basingstoke, England) at 37 °C. Bacterial cells were suspended in 5 mL of NaCl solution [0.85% (w/v)] until reach the cell density standardized on the no. 0.5 McFarland scale turbidity. Then, it was inoculated to the surface of MH agar, and sterile paper discs were placed on the inoculated agar surface. Each paper disc was moistened with 10 μ L of an individual lactobacilli cells preparation. Antimicrobial activity was measured by the size of bacterial growth inhibition halo around the discs after incubation at 37 °C for 48 h.

Antibiotic resistance

To evaluate the antibiotic resistance of the new lactobacilli isolates, the disc-diffusion method proposed by Charteris et al. (1998) was employed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2014). Twelve different antibiotics (Laborclin®, Pinhais, Brazil) were tested: ampicillin (10 μ g), ciprofloxacin (5 μ g), clindamycin (2 μ g), chloramphenicol (30 μ g), cefepime (30 μ g), erythromycin (15 μ g), gentamicin (10 μ g), oxacillin (1 μ g), penicillin

G (10 µg), sulfazotrim (25 µg), tetracycline (30 µg), and vancomycin (30 µg). *Escherichia coli* ATCC25922 was used as a quality control test. Lactobacilli isolates were classified as sensitive (S), moderately sensitive (MS), or resistant (R).

Antioxidant activity

Antioxidant activity was determined using two different assays: (1) 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) radical cation method, according to Pieniz et al. (2014) and (2) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity method, according to Brand-Williams et al. (1995). Antioxidant activity assays were performed with lactobacilli cell-free supernatants and intracellular extracts prepared and employed according to Pieniz et al. (2014).

Statistical analysis

Relationships among the technological and probiotic characteristics of the new lactobacilli isolates were determined by Principal Component Analysis (PCA) using PAST 3 software. The absolute values of the quantitative analysis were used to input data (acidification capacity, proteolytic activity, resistance of gastric and intestinal juices, and antioxidant activity). Results of qualitative assays (diacetyl production, antimicrobial activity, and antibiotic resistance) were converted into three coded values (0, 1, and 2) and used as input data.

Unweighted Pair Group Method using Arithmetic averages (UPGMA) analysis was performed for grouping the new 48 lactobacilli isolates based on their technological and probiotic characteristics.

Phylogenetic analyses were performed with a fragment of 1177 bp of *16S rRNA* gene using a Bayesian method. Alignments were performed using MUSCLE (Edgar 2004), implemented in MEGA 6.0 (Tamura et al. 2013) with default parameters and manually inspected. Bayesian analyses were performed using BEAST1.7 software (Drummond and Rambaut 2007). The model of nucleotide evolution used in all analyses was the GTR + I + G. Yule process was selected as a tree prior to Bayesian analysis, and 10,000,000 generations were performed using Markov Chain Monte Carlo (MCMC) algorithms. Tracer 1.5 (<http://beast.bio.ed.ac.uk/Tracer>) was used to check for convergence of the Markov chains and adequate effective sample sizes (> 200). Trees were visualized and edited using FIGTREE software (version 1.3.1).

Results

This work studied 48 new lactobacilli isolates, which were isolated from raw milk (38) and cheese (10). These bacterial isolates were closely related with ten species: *Lactobacillus*

paracasei (50% of the isolates), *L. parabuchneri* (15%), *L. pentosus* (13%), *L. zeae* (4%), *L. plantarum* (4%), *L. otakienensis* (4%), *L. casei* (4%), *L. harbinensis* (2%), *L. diolivorans* (2%), and *L. rhamnosus* (2%) (Table 1). Based on their technological and probiotic characteristics, these bacterial isolates were divided into five groups (Fig. 1a). Curiously, these bacterial isolates were also divided into five groups (clades) based on their phylogenetic relationships (Fig. 1b). However, the five groups in Fig. 1a, b are not related to each other.

Acidification potential (Δ pH and acidity) was recorded for milk, cheese whey, and permeate at 7 °C and 32 °C (Table S1). In milk, the Δ pH values at 7 °C after 24 h were lower (maximum Δ pH of 0.9 with CH131), while the Δ pH at 32 °C reached higher values (Δ pH maximum of 3.11 with CH135). Titratable acidity values reached 0.9% at 7 °C (CH131) and 0.97% at 32 °C (ML52). In cheese whey, the Δ pH values after 24 h reached 0.7 at 7 °C (CH131) and 2.63 at 32 °C (ML75b). Titratable acidity after 24 h reached 0.06% at 7 °C (CH131) and 0.53% at 32 °C (ML52). In permeate, the Δ pH after 24 h reached 0.66 (CH131), and the Δ pH at 32 °C reached 2.21 (CH131). The titratable acidity after 24 h reached 0.06% at 7 °C (ML26) and 0.55% at 32 °C (CH117). Based on these results, microorganisms CH131, CH135, and ML52 presented the greatest acidification capacities. Graphs for Δ pH and titratable acidity at both evaluated temperatures for the six most efficient lactobacilli isolates during the acidification process are shown in Fig. 2.

Halos formed by proteolytic activity ranged from 0.0 to 5.0 mm (Table S1). Ten lactobacilli (21%) presented no proteolytic activity, while 23 isolates (48%) presented small size halos (0.1–2.5 mm), and 15 isolates (31%) presented the highest size halos (2.5–5.0 mm). Lactobacilli ML34, ML12, ML88a, ML58, and ML04 presented the most promising results, reaching halo sizes up to 3.5 mm. Diacetyl production (Table S1) showed that 20 isolates (42%) produced high amounts of this compound, 5 isolates (10%) had an intermediate production, 11 isolates (23%) produced low amounts, and 12 isolates (25%) produced no diacetyl. Considering proteolytic activity and diacetyl production, isolates ML04, ML12, and ML88a showed the most promising results.

The resistance to simulated gastric and intestinal juices, and the survival rates of the strains under different pH values with or without bile salts addition are shown in Fig. 3 and Table S2. No tested lactobacilli were able to survive at pH 2 after 3 h of incubation (data not shown). Survival rates higher than 75% were observed by 32% of the lactobacilli isolates (15) at pH 2.5, while 69% of the isolates (33) survived at pH 3.0. All isolates (48) survived at incubation conditions lacking bile salts, while 42% of the isolates (20) survived at experimental conditions with bile salts. Most lactobacilli isolates were able to survive at pH 3.0 and without the addition of bile salts. At pH 2.5, isolate ML12 was the most resistant, followed by CH139, ML82, ML88a,

Table 1 Molecular identification of new *Lactobacilli* isolates

Strain ID	Accession	Sequence length (bp)	Closely related <i>Lactobacillus</i> spp. type strain	16S rRNA similarity (%)
ML 01	MF611797	1462	<i>L. paracasei</i> JCM 1171	98.07
ML 02	MF611798	1319	<i>L. paracasei</i> JCM 1171	97.87
ML 04	MF611799	1482	<i>L. parabuchneri</i> NBRC 107865	99.86
ML 07	MF611800	1483	<i>L. paracasei</i> JCM 1171	99.39
ML 10	MF611801	1223	<i>L. diolivorans</i> DSM 14421	99.51
ML 11	MF611802	1476	<i>L. paracasei</i> ATCC 25302	98.35
ML 12	MF611803	1348	<i>L. parabuchneri</i> NBRC 107865	97.63
ML 13	MF611804	1474	<i>L. paracasei</i> JCM 1171	98.76
ML 15	MF611805	1478	<i>L. paracasei</i> JCM 1171	99.45
ML 19	MF611806	1479	<i>L. parabuchneri</i> NBRC 107865	99.18
ML 21	MF611807	1444	<i>L. paracasei</i> ATCC 25302	99.79
ML 24	MF611808	1418	<i>L. paracasei</i> JCM 1171	97.72
ML 25	MF611809	1476	<i>L. casei</i> ATCC 393	99.25
ML 26	MF611810	1350	<i>L. parabuchneri</i> NBRC 107865	99.26
ML 28	MF611811	1469	<i>L. zae</i> ATCC 15820	99.79
ML 30	MF611812	1448	<i>L. harbinensis</i> DSM 16991	100.00
ML 31	MF611813	1350	<i>L. paracasei</i> JCM 1171	98.36
ML 33	MF611814	1480	<i>L. paracasei</i> JCM 1171	99.93
ML 34	MF611815	1468	<i>L. paracasei</i> JCM 1171	100.00
ML 36	MF611816	1350	<i>L. paracasei</i> JCM 1171	98.36
ML 47	MF611817	1468	<i>L. paracasei</i> JCM 1171	99.86
ML 52	MF611818	1464	<i>L. parabuchneri</i> NBRC 107865	99.59
ML 53	MF611819	1476	<i>L. otakiensis</i> JCM 15040	99.73
ML 58	MF611820	1477	<i>L. parabuchneri</i> NBRC 107865	99.80
ML 59	MF611821	1497	<i>L. zae</i> ATCC 15820	98.79
ML 60	MF611822	1328	<i>L. paracasei</i> JCM 1171	99.77
ML 62	MF611823	1481	<i>L. parabuchneri</i> NBRC 107865	98.35
ML 65B	MF611824	1464	<i>L. paracasei</i> JCM 1171	99.04
ML 66	MF611825	1483	<i>L. otakiensis</i> JCM 15040	99.66
ML 67	MF611826	1486	<i>L. casei</i> ATCC 393	98.98
ML 71	MF611827	1485	<i>L. paracasei</i> JCM 1171	99.46
ML 73	MF611828	1493	<i>L. plantarum</i> ATCC 14917	99.52
ML 75b	MF611829	1474	<i>L. paracasei</i> JCM 1171	99.04
ML 82	MF611830	1480	<i>L. pentosus</i> DSM 20314	99.32
ML 88a	MF611831	1464	<i>L. paracasei</i> JCM 1171	99.03
ML93p	MF611832	1459	<i>L. rhamnosus</i> JCM 1136	99.11
ML 99	MF611833	1467	<i>L. pentosus</i> DSM 20314	99.11
ML 104	MF611834	1404	<i>L. pentosus</i> DSM 20314	98.85
CH 112	MF611835	1457	<i>L. pentosus</i> DSM 20314	99.93
CH 114	MF611836	1483	<i>L. pentosus</i> DSM 20314	98.49
CH 117	MF611837	1347	<i>L. pentosus</i> DSM 20314	98.65
CH 123	MF611838	1468	<i>L. paracasei</i> JCM 1171	99.86
CH 131	MF611839	1492	<i>L. plantarum</i> ATCC 14917	98.70
CH 132	MF611840	1470	<i>L. paracasei</i> JCM 1171	98.56
CH 133	MF611841	1457	<i>L. paracasei</i> JCM 1171	99.93
CH 135	MF611842	1275	<i>L. paracasei</i> JCM 1171	98.04
CH 137	MF611843	1292	<i>L. paracasei</i> JCM 1171	97.52
CH 139	MF611844	1281	<i>L. paracasei</i> JCM 1171	98.44

Fig. 1 **a** Unweighted Pair Group Method using Arithmetic averages (UPGMA) analysis of 48 new lactobacilli isolates and their technological and probiotic characteristics. **b** Phylogenetic tree of the 48 lactobacilli isolates and 10 *Lactobacillus* reference strains inferred by

Bayesian analysis using 1177 bp of the *16S* rRNA gene. The significance of each branch is indicated at the branching points by posterior probability ≥ 0.90

and ML04. At pH 3.0, ML12, ML24, and ML71 showed 100% survival. The majority of the isolates remained at 10^7 and 10^8 CFU/mL in all tested pH values. However, ML15, ML59, ML93p, and CH123 isolates presented no viable cells at pH 2.5, and the same was observed to ML15 at pH 3.0. Lactobacilli isolates were slightly affected or unaffected in simulated conditions without the addition of bile salts: 17 isolates showed 100% survival, and none showed 0% survival. However, in the simulated conditions with the addition of bile salts, 13 strains presented no viable cells. Isolates ML33, ML75b, CH135, and CH139 showed 100% survival in simulated conditions with the addition of bile salts. It is important to highlight isolates ML12 and CH139, which showed high survival rates at pH 2.5 and 3, and in both with or without the addition of bile salts.

Evaluation of antibacterial activity of the new lactobacilli isolates (Fig. 4 and Table S3) showed that pathogens *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis*, and *Salmonella Typhimurium* were strongly inhibited by the pure lactobacilli cultures. Most of PS treatment showed weak antimicrobial action against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella enteritidis*. CS presented strong inhibition against the evaluated pathogens. Both NS and TS exhibited weak inhibition against the evaluated pathogens. Lactobacilli pellets showed strong inhibition against only *Salmonella Typhimurium*. The PG from ML07 and ML33 isolates showed the highest inhibition (halos > 10 mm) against the Gram-negative pathogens *Salmonella Typhimurium* and *Salmonella enteritidis*, and ML58 showed the highest inhibition potential

Values of Δ pH at 7°C in three different incubation times

Values of Δ pH at 32°C in three different incubation times

Values of titratable acidity at 7°C in three different incubation times

Values of titratable acidity at 32°C in three different incubation times

Fig. 2 Δ pH and titratable acidity values for the six most efficient lactobacilli isolates at 7 °C and 32 °C; and incubation period of 6, 9, and 24 h. Three different broth (milk, cheese whey, and permeate) were evaluated

(halos > 10 mm) against the Gram-positive pathogen *Listeria monocytogenes*. Isolate CH133 showed antibacterial activity (inhibition halos > 10 mm) against both Gram-positive and Gram-negative bacteria, specifically *Staphylococcus aureus*, *Salmonella Typhimurium*, and *Escherichia coli*.

Lactobacilli isolate ML25 showed high inhibition potential against all pathogen strains in all tested conditions (PG, PS, NS, TS, CS, and IE). ML31 exhibited similar results, but NS treatment with *Salmonella enteritidis*. ML36, ML47, CH123, and ML82 exhibited no inhibition activity against the tested pathogens. ML53 presented antimicrobial activity

only with PS treatment against *Salmonella enteritidis*, and CH117 isolate presented antibacterial activity only in pure culture treatment against *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis*, and *Salmonella Typhimurium*.

Table S4 shows the resistance profile of the new lactobacilli isolates against antibiotics. All isolates showed sensitivity to ampicillin, chloramphenicol, erythromycin, and tetracycline. Most of the isolates (41/85%) were sensitive to clindamycin, except one (2%, ML30) that showed resistance, while the others (6/13%) were moderately sensitive.

Fig. 3 Survival rates (%) of lactobacilli isolates at pH 2.5 and 3.0, without and with the addition of bile salts, after incubation at 37 °C

For penicillin G, there was a predominance of sensitive (27/56%) and moderately sensitive (19/40%) isolates, and only two (4%, ML04 and ML73) were resistant. In response to gentamicin, 25 lactobacilli (52%) were sensitive, and 23 (48%) were resistant. Furthermore, 30 (62.5%) isolates were sensitive to the antibiotic cefepime, and 18 (37.5%) were resistant.

Most of the lactobacilli isolates (44/92%) were resistant to oxacillin, while only one was moderately sensitive (2%—CH133), and three were sensitive (6%—ML15, ML52, and

ML104). Forty-two lactobacilli (88%) were resistant to vancomycin, and six isolates were sensitive (12%). Twenty-six isolates (54%) were resistant to ciprofloxacin, and 22 (46%) were sensitive. In addition, 30 isolates (63%) were resistant to sulfazotrim, 16 (33%) were sensitive, and two (4%) were moderately sensitive (ML01, ML99). Twenty-nine lactobacilli isolates (60%) were resistant to four different antibiotics.

Antioxidant activities of the intracellular extract and the supernatant of the lactobacilli isolates were evaluated by the ABTS and DPPH methods (Table S5). Application of the ABTS methodology to the supernatant resulted in values ranging from 0 to 39%. Three strains (6%, ML99, ML104, and CH117) presented no antioxidant activity, and four (8%, ML67, ML82, ML93p, and ML19) presented values ranging from 31 to 39%. Antioxidant activities of the intracellular extracts ranged from 0 to 14%. Three strains (6%, ML31, ML53, and CH131) exhibited high antioxidant activity values, and seven isolates (15%) presented no antioxidant activity. DPPH results indicated that supernatants presented antioxidant activities ranging from 0 to 34%, and the intracellular extract presented activities ranging from 0 to 6%. Seven isolates (15%) exhibited no antioxidant activity in supernatant, and 16 isolates (33%) in the intracellular extract. However, three lactobacilli (6%, ML01, CH133, and ML99) presented elevated supernatant antioxidant activity values.

Fig. 4 Principal Component Analysis (PCA) of the growth inhibition of important pathogens by lactobacilli isolates in pure bacterial growth (PG), pure supernatant (PS), neutralized supernatant (NS), heat-treated supernatant (TS), concentrated supernatant (CS), and

intracellular extract (IE). **a** *Staphylococcus aureus*; **b** *Escherichia coli*; **c** *Listeria monocytogenes*; **d** *Bacillus cereus*; **e** *Salmonella enteritidis*; **f** *Salmonella Typhimurium*

Correlation analysis among the technological and probiotic characteristics (Fig. 5) showed a general profile of our new lactobacilli isolates. This analysis indicates that acidification potential (% acid and Δ pH) is highly dependent on the incubation temperature and not on the incubation broth (Fig. 5a). Antioxidant activity of the intracellular extract was correlated with diacetyl production (Fig. 5b), and these three characteristics have an inverse correlation with lactobacilli resistance to intestinal juice with bile salts addition (RIJ+). Bacterial resistance to the antimicrobials ciprofloxacin, gentamicin, and vancomycin was correlated, while resistance to cefepime, sulfazotrim, and clindamycin was also correlated (Fig. 5c).

Discussion

Lactobacillus genus comprises several lactic acid bacteria (LAB) with high technological and probiotic potential (Toualbia et al. 2018). Evaluation of the acidification potential, proteolytic activity and diacetyl production contributes to the technological characterization of these bacterial isolates. Beresford et al. (2001) concluded that a bacterium that produces acid to reduce the milk pH below 5.3 after 6 h of incubation at 30 °C is considered a good inoculum for fermented foods. In the present work, we verified this acidification potential with the isolates CH112 and CH131. Proteolytic activity and diacetyl production (resulting from citrate metabolism) are important for industrial processes, because these attributes contribute to the aroma, flavour, and consistency of fermented foods (Rincon-Delgadillo et al. 2012). Tullini et al. (2015) showed that most of the studied LAB presented proteolytic activity ($n=38$, 79%), high diacetyl production ($n=20$, 42%), and were capable of hydrolysing milk proteins, increasing the food digestibility, and contributing to the production of desirable flavours. Milk proteins are source of essential amino acids for LAB, stimulating their growth and consequently contributing to the taste of fermented dairy products (Lopez-Kleine and Monnet 2011). Regarding the diacetyl production, in this study, 42% of lactobacilli isolates were able to produce diacetyl, indicating an interesting technological potential to the production of fermented foods.

Tests to measure resistance to the gastric acidity present in bile salts are often performed with microorganisms that present probiotic potential. This work showed that studied lactobacilli generally present high survival when inoculated in gastric juice (pH 2.5). The survival pattern was strain-specific and differed among bacterial isolates belonging to the same species. Similar pattern was observed by Zanoni et al. (2008) and Meira et al. (2012), who also found that this profile is affected by environmental conditions.

The ability to inhibit the growth of pathogenic bacteria is an important characteristic for probiotic microorganisms (Monteagudo-Mera et al. 2012). Some LAB strains are able to produce antimicrobial substances that inhibit the growth of pathogenic and sporulating microorganisms (Herreros et al. 2005). It has been previously noted that metabolic sub-products of LAB, such as lactic acid, organic acids (Caplice and Fitzgerald 1999), acetaldehyde, hydrogen peroxide, carbon dioxide, diacetyl, and polysaccharides, have antimicrobial effects (Rodríguez et al. 2003). This study evaluated antibacterial activity aiming to show their inhibitory capacity. PG and CS presented the greatest inhibition activity against pathogens. Sixty percent of lactobacilli PG inhibited the development of pathogenic *Salmonella enteritidis*. The lowest antimicrobial effects was observed in NS treatment. Ren et al. (2014) suggested that neutralization process could inactivate antibacterial substances.

Probiotic microorganisms must be safe for human consumption. Some probiotic strains can show resistance to specific antibiotics to survive in an environment treated with antibiotic therapy. An increasing attention has been given to antimicrobial use in farm animal feeding, which can act as precursors of antibiotic-resistant microorganisms in humans (Solieri et al. 2014). All lactobacilli studied in this work were sensitive to ampicillin, chloramphenicol, erythromycin, and tetracycline. Testore et al. (2002) and Gevers et al. (2003) showed that resistance of *Lactobacillus* spp. to these antibiotics is associated with acquired resistance (by horizontal gene transfer). Most of our lactobacilli were also resistant to oxacillin, vancomycin, ciprofloxacin, and sulfazotrim. Danielsen and Wind (2003) and Herreros et al. (2005) demonstrated that *Lactobacillus* spp. present natural, intrinsic, non-transmissible resistance to these antibiotics. *Lactobacillus* spp. resistance to ciprofloxacin has also been widely documented (Charteris et al. 1998; Katla et al. 2001; Danielsen and Win 2003). However, the origin of this resistance is not clear, and some studies indicate that it may be an intrinsic bacterial characteristic (Monteagudo-Mera et al. 2012). Most of the LAB do not have the folic acid synthesis pathway, which is the target of sulfonamides and trimethoprim. Therefore, they may also have an intrinsic resistance to the antibiotic sulfazotrim (Katla et al. 2001).

Antioxidants' production by LAB may decrease oxidative stress in host cells (Zanoni et al. 2008; Wang et al. 2009). Antioxidative factors also prevent cardiovascular diseases, diabetes, and ulcers in the gastrointestinal tract (Kaushik et al. 2009). Our results showed that the antioxidant potential observed in bacterial cell supernatants was higher than in intracellular extract. According to Lin and Yen (1999), the intracellular extracts of LAB chelate metal ions and eliminate reactive oxygen species, but it presents reduced activity. The antioxidant activity observed in supernatant culture is advantageous considering the free radical transport in cells

Fig. 5 Principal Component Analysis (PCA) of the technological and probiotic characteristics of lactobacilli isolates. **a** Proteolytic activity, diacetyl production, titratable acidity (% acid) and pH variation (Δ pH); **b** antioxidant activity of the supernatant (AAS), antioxidant activity of the intracellular extract (AAIE), resistance to intestinal juice with (RIJ+) and with no addition of bile salts (RIJ-), resistance to gastric juice (RGJ) at two pH values (2.5 and 3.0), proteolytic activity and diacetyl production; and **c** Intensity of the resistance to 12 antibiotics

(Pieniz et al. 2015). The antioxidant potential observed in the lactobacilli studied in this work suggests that they may be useful for the development of functional foods acting as antioxidants to eliminate free radicals from host cells.

The data presented here show that some lactobacilli isolated from raw milk and cheese from southern Brazil have technological characteristics and probiotic potential to create foods with differentiated flavours and functional product characteristics. Principal Component Analysis (PCA) was used to investigate correlations among technological and probiotic characteristics, and identify new promising lactobacilli isolates for exploring their characteristics. PCAs presented in Figs. 4 and 5 explain the high percentage of variability (PC1 + PC2). Such high percentage suggests a homogeneous distribution of the lactobacilli isolates with important and significant differences. Similar to Perricone et al. (2014), Angmo et al. (2016), and Kumari et al. (2016), technological and probiotic characteristics of the *Lactobacillus* spp. presented no pattern for the genus (as observed in PCA analysis) and probiotic and technological potential of new lactobacilli isolates are strain specific. Five strains (ML01, ML04, ML12, ML88, and CH139) showed both technological and probiotic characteristics. Characterization of these microorganisms is an important first step to stimulate their application in fermented foods or as probiotics, benefiting the population health.

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Compliance with ethical standards

Conflict of interest The authors declare that they do not have any conflict of interest.

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