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Queijo coalho como fonte de bactérias ácido láticas probióticas

Coalho cheese as source of probiotic lactic acid bacteria

Queso coalho como fuente de bacterias de ácido láctico probiótico

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Resumo

O objetivo deste estudo foi caracterizar o potencial probiótico de 24 cepas de bactérias ácido láticas (BAL) isoladas de queijo Coalho artesanal do Pernambuco, Brasil, utilizando testes in vitro. Foram avaliadas a resistência ao trato gastrointestinal (TGI), atividade antimicrobiana contra patógenos intestinais, capacidade de autoagregação e coagregação, hidrofobicidade celular, atividade de ß-galactosidase, atividade de desconjugação de sais biliares pela produção de bile sal hidrolase (BSH) e sensibilidade a antibióticos. Das 24 cepas, 22

permaneceram viáveis após passagem pelo TGI simulado. Duas cepas inibiram o crescimento de *Listeria monocytogenes* e duas inibiram a *Escherichia coli*. A taxa de autoagregação variou entre 27% e 96%, e as cepas foram capazes de coagregar com *Staphylococcus aureus* e *E. coli*, atingindo níveis de 58% e 47%, respectivamente. O percentual de hidrofobicidade variou entre 5% e 57%. Quatro cepas foram capazes de produzir BSH. Uma BAL foi capaz de produzir até 604 unidades Miller de ß-galactosidase. Todas as cepas foram sensíveis a cinco antibióticos e apenas duas foram resistentes à vancomicina (30µg) e norfloxacina (10g). As cepas que foram capazes de ultrapassar todas as barreiras com a redução de apenas um ciclo logarítmico e as que foram capazes de produzir ß-galactosidase foram identificadas pela análise da sequência de 16S rRNA como *Lactococcus lactis* subsp. *Lactis*, *Enterococcus durans* e *Enterococcus faecium*. Todas as BAL avaliadas apresentaram características probióticas promissoras. Cepas identificadas como *L. lactis* subsp. *Lactis* foram selecionadas para estudos envolvendo seu potencial tecnológico a fim de avaliar o possível uso desses microrganismos em um produto funcional.

Palavras-chave: Produtos lácteos; Queijo artesanal; Probióticos.

Abstract

The aim of this study was to characterize the probiotic potential of 24 lactic acid bacteria (LAB) strains isolated from artisanal Coalho cheese from Pernambuco, Brazil by in vitro tests. The gastrointestinal tract (GIT) resistance, antimicrobial activity against intestinal pathogens, autoaggregation and coaggregation capacity, cell hydrophobicity, ß-galactosidase activity, deconjugate bile salt activity for the production of bile salt hydrolase (BSH), and the sensitivity to antibiotics were evaluated. Of the 24 strains, 22 remained viable to a simulated GIT. Two LAB inhibited the growth of *Listeria monocytogenes* and two inhibited *Escherichia coli*. The autoaggregation rate ranged from 27% to 96%, and the strains were able to coaggregate with *Staphylococcus aureus* and *E. coli* reaching levels between 58% and 47%, respectively. The hydrophobicity percentage ranged from 5% to 57%. Four strains were able to produce BSH. One LAB was able to produce up to 604 Miller units of ß-galactosidase. All strains were sensitive to five antibiotics and only two were resistant to vancomycin (30µg) and norfloxacin (10g). LAB strains which were able to overcome all barriers with a reduction of only one log cycle and LAB strains which were able to produce ß-galactosidase were identified by 16S rRNA sequence analysis as *Lactococcus lactis* subsp. *Lactis*, *Enterococcus durans*, and *Enterococcus faecium*. The evaluated LAB showed promising probiotic characteristics. Strains identified as *L. lactis* subsp. *Lactis* were selected for studies involving

their technological potential to investigate the possible use of these microorganisms into a functional product.

Keywords: Dairy products; Artisanal cheese; Probiotics.

Resumen

El objetivo de este estudio fue caracterizar el potencial probiótico de 24 cepas de bacterias del ácido láctico (BAL) aisladas del queso Coalho artesanal de Pernambuco, Brasil, mediante pruebas in vitro. Se evaluó la resistencia al tracto gastrointestinal (GIT), la actividad antimicrobiana contra los patógenos intestinales, la capacidad de autoagregación y coagregación, la hidrofobicidad celular, la actividad ß-galactosidasa, la actividad de desacoplamiento de la sal biliar con la producción de hidrolasa de sal biliar (BSH) y la sensibilidad. Antibióticos. De las 24 cepas, 22 permanecieron viables después de pasar por el TGI simulado. Dos cepas inhibieron el crecimiento de *Listeria monocytogenes* y otras dos inhibieron *Escherichia coli*. La tasa de autoagrupación varió entre 27% y 96%, y las cepas pudieron agruparse con *Staphylococcus aureus* y *E. coli*, alcanzando niveles de 58% y 47%, respectivamente. El porcentaje de hidrofobicidad varió entre 5% y 57%. Cuatro cepas pudieron producir BSH. Una BAL fue capaz de producir hasta 604 unidades Miller de ß-galactosidasa. Todas las cepas fueron sensibles a cinco antibióticos y solo dos fueron resistentes a la vancomicina (30 μg) y la norfloxacina (10 g). Las cepas que pudieron superar todas las barreras con la reducción de un solo ciclo logarítmico y las que pudieron producir ß-galactosidasa fueron identificadasmediante el análisis de la secuencia de ARNr 16S como *Lactococcus lactis* subsp. *Lactis, Enterococcus durans* y *Enterococcus faecium*. Todos las BAL evaluadas mostraron características probióticas prometedoras. Cepas identificadas como *L. lactis* subsp. *Lactis* se seleccionó para estudios que implican su potencial tecnológico para evaluar el posible uso de estos microorganismos en un producto funcional.

Palabras clave: Productos lácteos; Queso artisanal; Probióticos.

1. Introduction

The word ‘probiotics’ originates from the Greek word ‘for life’. According to FAO/WHO guidelines, probiotics are defined as live organisms which confer a health benefit on the host when administered in adequate amounts. Probiotics are also defined as live bacteria which contribute to the regulation of immune responses and have beneficial effects on the host. Resistance to enteric pathogens, aid in lactose digestion, anti-colon cancer effect,

small bowel bacterial overgrowth, and immune system modulation are some of the beneficial actions of probiotic bacteria (Lee and Salminen, 2009).

Certain criteria need to be met by a bacterium to qualify as a probiotic: it must be harmless for ingestion (safe), able to colonize the gut epithelium, and especially be able to resist harsh conditions found in the gastrointestinal tract (gastric acidity, bile salts, pepsin, pancreatin, and other enzymes) (Lee and Salminem, 2009). Therefore, knowledge of these characteristics is important for evaluating possible positive and negative effects of probiotic consumption.

In the past, the GIT was considered the main potential source of probiotic bacteria (Espinoza & Navarro, 2010). However, the scientific community has focused attention on fermented foods and recognized the autochthonous lactic microbiota of different foods with probiotic potential. Thus, the search for new probiotic strains derived from dairy (Nuryshev, Stoyanova & Netrusov, 2016; Haghshenas, et al. 2017) and non-dairy (Saito et al. 2014; Mortezaei et al. 2020) is justified by the possibility of detecting strains with benefits to human health and with good technological performance.

Coalho cheese is a semi-hard cheese, typically produced and widely consumed in the Northeast of Brazil by coagulation of milk with rennet or other appropriate coagulating enzymes, complemented or not by the action of selected lactic acid bacteria (LAB) (Brasil, 2001). Several studies have isolated autochthonous LAB in this cheese (Santos et al. 2015; Medeiros et al. 2016; Bruno et al. 2017) searching for strains with technological and/or probiotic features, as most probiotic bacteria belong to LAB group. The main representative LAB are Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella (Jay, Loessner & Golden, 2005).

Thus, the aims of our research were to characterize the probiotic potential of 24 autochthonous LAB strains isolated from Coalho cheese produced in Pernambuco state, Brazil.

2. Materials and Methods

This research was based on a laboratory study. Lactic acid bacteria (LAB) strains isolated from artisanal Coalho cheese were studied in the research. The results obtained were qualitatives and quantitatives with statistical analysis (Pereira et al.,2018).

2.1 Bacterial strains and culture conditions

LAB strains isolated from artisanal Coalho cheese from Pernambuco state, Brazil, were stored (-80ºC) in Man Rogosa Sharpe (MRS, Oxoid) broth supplemented with 15% (v/v) glycerol. Prior to assays, the strains were transferred to MRS broth and cultivated at 37ºC/24h at least three times in aerobic conditions.

2.2 Resistance to simulated gastrointestinal tract

A sequence of tests simulating gastrointestinal conditions were used according to Burns et al. (2011) with modifications to verify the strains' tolerance to the GIT. LAB were inoculated into saliva solution ($CaCl_2$ 0.22 g L^{-1}, NaCl 16.2 g L^{-1}, KCl 2.2 g L^{-1} and $NaHCO_3$ 1.2 g L^{-1}) containing bovine pepsin 0.3% w/v (Sigma-Aldrich). The pH was quickly lowered to 2.0 immediately after mixing, with HCl 5N and incubated in a water bath for a period of 90 min at 37°C. After simulated saliva-gastric digestion, 1 mL of the sample was centrifuged (4.000 g, 5 min, 5°C), the supernatant was removed, and the pellet was washed twice with buffered phosphate saline (PBS) solution (pH 7.4) and resuspended to the original volume in 1% (w/v) bovine bile (Sigma-Aldrich) at pH 8.0. The cell suspension was incubated in a water bath for 10 min/37°C. Next, it was centrifuged and the cells were washed as described above and resuspended to the original volume in 0.3% (w/v) bovine bile and 0.1% (w/v) pancreatin (Sigma-Aldrich) at pH 8.0. The cell suspension was then again incubated in a water bath at 37°C/90min. Cell viability was monitored before the beginning of the first test and at the end of each stage by plating on MRS agar at 37°C/48h. The test was performed in triplicate.

2.3 Antimicrobial activity

Antimicrobial activity of LAB against intestinal pathogens (*Listeria monocytogenes* ATCC 7644, *Salmonella thyphi* ATCC 6539, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218) was evaluated by the well-diffusion agar assay according to Vinderola et al. (2008) with modifications. Cell-free supernatants (CFS) of LAB were obtained by centrifugation of overnight cultures (12000g, 5min, 5ºC), and sterilization by filtration through a 0.22mm pore filter (Millipore). Tubes with 20 mL of nutrient agar (Merck) (for *S. typhi*, *S. aureus* and *E. coli*), or Brain heart infusion (BHI) agar (Merck) (for

L. monocytogenes), were melted and cooled to 45ºC, inoculated (2%, v/v) with pathogenic cultures (OD_{560} 0.8), vigorously homogenized and poured onto Petri dishes. Wells of 10mm in diameter were made in the agar layer of plates containing pathogens, and 180µL of the CFS from each strain were placed in a well. Plates were incubated overnight at 37ºC and the diameters of the inhibition halos were measured.

2.4 Autoaggregation and coaggregation with pathogens strains

Autoaggregation and coaggregation assays were performed according to Kos et al. (2003), but modified as follows: cell suspension of each LAB (approximately 10^8 $CFU.mL^{-1}$) was prepared to determine autoaggregation and coaggregation. Tubes containing only the LAB and tubes with LAB and pathogenic bacteria (*S. aureus* ATCC 25923 and *E. coli* ATCC 35218) were vigorously mixed (10 seconds) and then left at room temperature (20ºC) for 5 hours. An aliquot of the cell suspension was picked up each time and absorbance (OD_{600}) was measured. The autoaggregation percentage is expressed as $1 - (A_t/A_0) * 100$, where A_t represents the absorbance at time t = 1, 2, 3 or 4 h and A_0 the absorbance at t = 0. The coaggregation percentage was calculated using the equation of Handley et al., (1987) as: $[1-2A_{mix}/(A_{probio}+ A_{pathog})]\times100$, where A_{probio} and $A_{pathogen}$ represented each of the two strains in the control tubes and A_{mix} denoted the mixture.

2.5 Hydrophobicity

The cell hydrophobicity was determined according to Vinderola and Reinheimer (2003). Cultures of the strains were harvested in the stationary phase by centrifugation (12000*g*, 5min, 5ºC), washed twice in 50mM of K_2HPO_4 (pH 6.5) buffer and resuspended in the same buffer. The cell suspension was adjusted to a 560nm absorbance value of approximately 1.0 (OD_{560} 1.0) with the buffer and 3 ml of the bacterial suspensions were put in contact with 0.6ml of n-hexadecane (Merck Schuchardt, Germany) and vortexed for 120s. After separating the two phases, the aqueous phase was carefully removed and the absorbance was measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H %), which was calculated with the formula $H\% = [(A_0 – A)/A_0]*100$, where A_0 and A are the absorbance before and after extraction with n-hexadecane, respectively. Assays were performed in triplicate.

2.6 ß-galactosidase activity

ß -galactosidase activity (ß - gal) in whole cells was determined according to the method of Miller (1972) modified by Vinderola and Reinheimer (2003). Overnight strain cultures were harvested in the stationary phase by centrifugation (12000*g*, 5min, 5ºC), washed twice in 60 mM $Na_2HPO_4.7H_2O$/40 mM NaH_2PO_4 buffer (pH 7.0), inoculated (1% v/v) in MRS-lac broth and incubated at 37ºC for 24h. After the incubation period, cells were harvested and washed twice as previously described, and 560nm absorbance was adjusted to approximately 1.0 (OD_{560} 1.0) with the same buffer. One milliliter of the cell suspension was permeabilized with 50µL of toluene/acetone (1:9 v/v) solution, vortexed for 7 min and immediately assayed for ß-galactosidase activity. An aliquot of 100 µl of the permeabilized cell suspension was placed in a tube and 900 µl of phosphate buffer and 200µl of o-nitrophenyl-b-D-galactopyranoside (ONPG, Sigma) (4 mg ml^{-1}) were added. Tubes were placed into a water bath (37ºC) for 15 min. Then, 0.5 ml of 1M Na_2CO_3 was added to each tube to stop the reaction. Absorbance values at both 420 and 560nm were recorded for each tube. Enzymatic activity (ß-gal; Miller units) was calculated as follows: 1000x [(A_{420}*1.75*A_{560}^b)/(15min*1ml*A_{560}^a)], where A_{560}^a was the absorbance just before assay and A_{560}^b was the absorbance value of the reaction mixture.

2.7 Bile salt deconjugation ability

The strain activity to produce bile salt hydrolase (BSH) was determined according to Zago *et al.* (2011). Bile salt plates were prepared by adding 0.5% (w/v) of sodium salts (Sigma) of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC) and glicodeoxycholic acid (GDC) to MRS agar containing 0.37 g/L $CaCl_2$. The strains were streaked onto the media and the plates were anaerobically (Anaerocult® A - Merck Millipore) incubated at 37ºC for 72 h. The presence of halos around colonies or white opaque colonies indicated BSH activity. *Enterococcus faecium* ATCC 6569 was used as BSH-positive strain. The inoculum of each strain in MRS without supplementation was included as a negative control.

2.8 Antibiotic resistance

The disc diffusion method was used to evaluate the antibiotic susceptibility of LAB. Tests were done according to the criteria of the National Committee of Clinical Laboratory Standards (NCCLS, 1997) with modifications. Cells were grown in MRS broth at 37ºC for 18h to obtain a density of approximately 10^7 cells/ml. The cell suspension was inoculated in MRS agar plates with the aid of a sterile "swab". Antibiotic discs were dispensed on to the media and incubated at anaerobic conditions at 37ºC for 24h. Seven discs (LABORCLIN®) of antibiotics were tested: ampicillin (AM 10µg), erythromycin (E 15µg), vancomycin (VA 30µg), chloramphenicol (C 30µg), tetracycline (TE 30µg), streptomycin (S 300µg), and norfloxacin (NOR 10µg). Inhibition-zone diameters were measured after incubation and susceptibility is expressed in terms of resistance (R), moderate susceptibility (MS), and susceptibility (S), based on CLSI M100-S15 guidelines for the standard strains (NCCLS). Each experiment was performed in triplicate.

2.9 Identification of LAB strains

The identification of LAB strains was determined at the Stab Vida (Caparica, Portugal). Total DNA of isolates was obtained from cell culture stored on FTA™ indicating Micro card. The identity of isolates was analysed by PCR amplification of the 16S rDNA gene and DNA Sanger sequencing (sense and antisense) of the amplified product. The identity of isolates was checked by nucleotide BLAST of the NCBI database (www.ncbi.nlm.nhi.gov/blast)

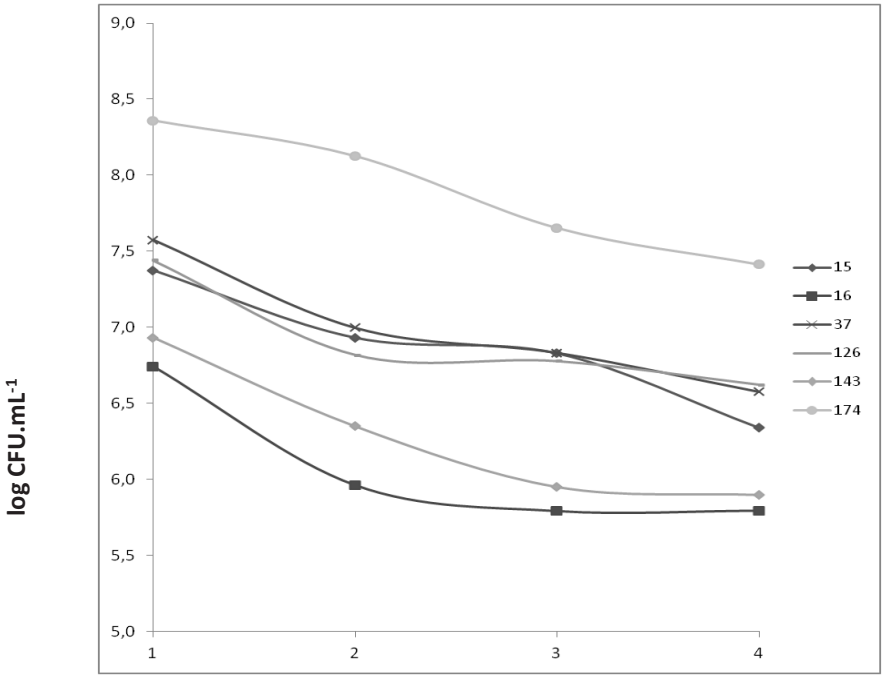
3. Results and Discussion

3.1 Resistance to simulated gastrointestinal tract

The evaluated LAB strains showed high resistance to the combined stress at the various steps of the simulated gastrointestinal tract. All strains survived simulated gastric juice, exposure to pH 2.5 and simulated duodenal juice (Table 1). However, only 22 LAB remained viable after the passage to simulated GIT conditions. Six strains (15, 16, 37, 126, 143 and 174) were able to overcome all barriers with a reduction until one log cycle (Figure 1).

Several authors have reported the GIT resistance of strains isolated from different sources. Saito et al. (2014) concluded that lactobacilli strains isolated from the fermentation of cocoa survived the barrier of simulated GIT and Lactobacillus fermentum showed higher resistance than Lactobacillus plantarum to the simulated gastrointestinal digestion at pH 3, losing only approximately one log order of cell viability. Nejati and Oelschlaeger (2016) also observed that *Lactococcus lactis* isolated from traditional Iranian dairy products had better survival after exposure to simulated gastrointestinal tract stresses in comparison to the control Lactobacillus rhamnosus GG probiotic.

Figure 1. Cell counts (log CFU.mL^{-1}) of LAB in initial count (1) after simulated gastric digestion at pH 2.0 in saliva gastric exposure (2), after exposure to simulated duodenal juice (3), and after exposure to simulated ileo juice (4). Values are means of 3 replicates.



Source: Elaborated by the authors.

In our study, major decreases in the viability of strain cells (around 4 log orders) were registered after the incubation with bile, with cell counts at the end of the simulated GIT conditions between 10^3 and 10^5 CFU.mL^{-1}. Han, Kong, Chen, Sun and Zhang (2017) reported poor bile resistance for *L. acidophilus*. All LAB strains in their study grew on the plates containing 0.3% bile salts. The strains with shorter lag time (less than 4 h) grew on plates with 1% bile salts. *L. pentosaceus* specifically survived in 2% bile salts.

3.2 Antimicrobial activity

Cell-free supernatants from two microorganisms (strains 128 and 155) showed antimicrobial activity against *L. monocytogenes* ATCC 7644. The inhibitory zones were 5.3 mm (strain 128) and 8.1 mm (155). Another two strains (64 and 174) inhibited the growth of *E. coli* ATCC 35218, with inhibition halos of 4.2 mm (strain 64) and 6.3 mm (strain 174). *Salmonella typhi* ATCC 6539 and *S. aureus* ATCC 25923S were not inhibited by the evaluated LAB.

Development of mechanisms to survive in competition with other microorganisms in the GIT complex is a desirable feature in probiotic strains. Cabral *et al*. (2016) also observed antagonistic activity of the lactic acid bacteria isolated from artisanal and industrial Coalho cheese samples against *Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus*. Bruno *et al*. (2017) reported inhibition zones in plates with *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* sp. In their study, seven LAB isolated from Coalho cheese showed inhibition halos around the well. All diameters of the produced halos were under 10 mm.

3.3 Autoaggregation and coaggregation

The evaluated LAB showed autoaggregation percentage ranging from 27.27% (strain 99) to 96.43% (strain 155) (Table 1). The tested strains showed the best coaggregation result with *S. aureus*, which reached levels of 58.33% of coaggregated bacteria (strain 42), and for *E. coli* with a coaggregation result of 47.83% with strain 46 (Table 1).

Table 1. LAB counts (log CFU.mL-1) in simulated GIT (Values are means (±SD) of 3 replicates). Autoaggregation and coaggregation percentage.

Strain	Counts (log CFU) in simulated TGI[a]					Autoaggregation[c]	Coaggregation[d]	
	1	2	3	4	Δ log cfu[b]		*E. coli*	*S. aureus*
15	7.4	6.9	6.9	6.4	1.0	61.23 ± 2.81	34.13 ± 1.37	36.74 ± 3.10
16	6.7	6.0	5.8	5.8	0.9	89.92 ± 1.36	46.67 ± 0.00	27.78 ± 6.94
17	6.4	4.6	3.9	3.6	2.8	76.39 ± 2.64	35.16 ± 4.68	28.19 ± 0.33
37	7.6	7.0	6.8	6.6	1.0	75.53 ± 2.51	44.43 ± 4.01	28.19 ± 0.33
40	7.7	6.5	4.6	4.4	3.3	72.96 ± 0.69	36.00 ± 0.00	52.22 ± 1.92
42	6.9	5.6	5.0	4.9	2.0	77.95 ± 3.61	49.76 ± 4.14	58.33 ± 4.22
46	7.9	5.9	4.8	4.7	3.2	81.87 ± 0.63	47.83 ± 0.00	56.04 ± 1.90
60	7.9	4.8	4.1	3.9	4.0	88.00 ± 1.53	46.30 ± 3.21	24.52 ± 3.19
64	8.1	6.7	4.0	3.8	4.3	83.43 ± 1.52	41.90 ± 1.65	30.66 ± 0.19
68	7.9	6.4	4.5	4.1	3.8	88.45 ± 2.06	15.74 ± 5.61	46.02 ± 2.75
76	8.0	6.7	4.8	4.2	3.8	75.81 ± 1.47	53.19 ± 2.05	44.42 ± 1.99
98	7.6	3.8	1.3	0.0	7.6	49.19 ± 0.70	29.72 ± 2.65	15.83 ± 2.67
99	7.4	5.7	5.0	4.1	3.3	27.27 ± 0.37	7.94 ± 5.50	23.75 ± 0.60
106	7.6	6.9	5.6	5.1	2.5	76.27 ± 2.11	39.11 ± 3.47	49.97 ± 1.56
110	6.7	5.6	4.0	3.1	3.0	86.54 ± 2.20	48.76 ± 3.62	43.30 ± 2.90
117	7.6	3.0	3.1	3.0	4.6	92.13 ± 5.16	40.21 ± 6.01	19.01 ± 2.36
126	7.4	6.8	6.8	6.6	0.8	75.68 ± 3.77	14.07 ± 5.13	53.63 ± 2.51
128	6.7	5.5	4.6	3.5	3.2	84.07 ± 1.07	24.85 ± 4.20	45.38 ± 2.39
143	6.9	6.3	5.8	5.9	1.0	90.12 ± 1.57	7.89 ± 2.82	20.58 ± 1.00
145	7.6	5.8	3.8	3.1	4.5	92.48 ± 3.19	30.16 ± 2.75	15.49 ± 2.32
147	8.0	3.9	1.6	0.0	8.0	77.02 ± 2.79	44.97 ± 0.84	46.38 ± 3.05
155	7.9	4.9	4.7	3.1	4.8	96.43 ± 3.97	39.12 ± 5.08	17.83 ± 0.31
158	7.1	5.4	3.6	3.1	4.0	93.65 ± 1.72	47.62 ± 4.12	45.07 ± 3.39
174	8.4	8.1	7.7	7.3	0.9	88.58 ± 1.72	13.13 ± 2.40	24.21 ± 0.69

a: 1: Initial cell counts; 2: after simulated gastric digestion at pH 2.0 in saliva gastric exposure; 3: after exposure to simulated duodenal juice; 4: after exposure to simulated ileo juice. **b**: range between the initial and final count (after exposure to simulated GIT). **c**: Autoaggregation percentage; **d**: Coaggregation percentage.
Source: Elaborated by the authors.

Our results are similar to results reported by several studies with high percentage of autoaggregation and moderate coaggregation. Janković, Frece, Abram and Gobin (2012) investigated the aggregation and coaggregation ability of three potential probiotic strains of *Lactobacillus plantarum* and observed that the autoaggregation rate after 24 hours of broth cultivation was at least 80%, and coaggregation with *E. coli, S. Typhimurium* and *L. monocytogenes* was 41.5%, 40.5%, 37.4%, respectively. Han *et al.* (2017) observed that the highest auto-aggregation percentage at 24 h were demonstrated by *L. brevis* (80.5%) and *L. acidophilus* (77.8%), with no significant difference between them.

3.4 Hydrophobicity

The evaluated LAB presented hydrophobicity levels ranging from 5.13% (strain 155) to 57.61% (strain 15) (Table 2). Nejati and Oelschlaeger (2016) found similar results and reported cell hydrophobicity of *Lactococcus lactis* strains isolated from Traditional Iranian

Dairy Products ranging from 5.13 to 83.68 %. In both studies, evaluation of hydrophobicity of the 8 isolated strains revealed considerable differences between the strains.

3.5 ß-galactosidase activity

A total of 20 tested strains were able to produce the ß-galactosidase enzyme. The ß-galactosidase values found ranged from 2 to 604 Miller units (strain 46) (Table 2). Shukla, Iliev and Goyal (2014) reported a β-galactosidase activity of 94.24 Miller units for *Leuconostoc mesenteroides*.

Table 2. Probiotic characteristics for lactic acid bacteria (mean±standard deviation, n=3).

Strain	%H[a]			ß Gal[b]		
15	57.61	±	2.13	6.34	±	0.88
16	45.91	±	3.80	1.67	±	0.33
17	39.05	±	2.06	72.90	±	7.91
37	5.83	±	0.51	21.39	±	6.43
40	8.53	±	0.97	2.00	±	0.72
42	39.44	±	2.07	2.62	±	0.53
46	49.18	±	3.24	604.88	±	33.25
60	51.13	±	1.64	131.44	±	9.87
64	31.22	±	2.90	5.11	±	0.42
68	9.94	±	2.36	9.32	±	0.41
76	52.20	±	0.78	0.54	±	0.19
98	9.56	±	1.87	3.94	±	1.62
99	7.70	±	0.63	44.88	±	6.52
106	23.23	±	2.07	219.72	±	8.53
110	45.92	±	2.10	41.62	±	1.62
117	47.46	±	2.82	51.22	±	3.87
126	10.99	±	0.90	38.84	±	1.94
128	26.63	±	1.42	31.93	±	0.36
143	10.46	±	1.80	0.00	±	0.00
145	6.73	±	2.24	0.90	±	0.57
147	4.93	±	0.77	3.83	±	0.23
155	5.13	±	0.64	14.33	±	0.58
158	6.13	±	0.99	0.32	±	0.31
174	5.86	±	1.60	25.11	±	5.25

a: Hydrophobicity percentage. **b**: β-galactosidase activity in Miller units.
Source: Elaborated by the authors.

On the other hand, the ß-galactosidase activity values observed for the evaluated strains were lower than those reported by Son *et al.* (2017), where all the LAB strains showed higher ß-galactosidase enzyme activity when grown in MRS broth containing 1% lactose than when grown in broth without 1% lactose, thereby indicating that the addition of 1% lactose

could increase b-galactosidase enzymatic activity. *L. plantarum* showed the highest b-galactosidase enzyme activity in the study by Son et al. (3320.99 Miller units).

3.6 Bile salt deconjugation ability

All evaluated microorganisms were able to grow in the presence of sodium taurodeoxycholate, taurocholic acid and glycocholic acid, but only 17 strains were able to show full resistance to sodium glycodeoxycholate (Table 3). The strains 46, 60, 106 and 128 showed white opaque colonies after growth in MRS-TDCA, evidencing the ability to deconjugate sodium taurodeoxycholate. Strains 106 and 128 hydrolyzed sodium glycodeoxycholate detected by halo formation around colonies after growth in MRS-GDCA. Zago *et al.* (2011) reported similar results in their study. All strains evaluated for these authors demonstrated the ability to hydrolyze the sodium glycodeoxycholate and sodium taurodeoxycholate.

3.7 Antibiotic resistance

All tested strains were susceptible (S) to five antibiotics: ampicillin (AM 10 µg), erythromycin (E 15 µg), chloramphenicol (C 30 µg), tetracycline (TE 30 µg) and streptomycin (S 300 µg). Only two strains (37 and 98) were resistant (R) to vancomycin (VA 30 µg) and norfloxacin (NOR 10 µg) (Table 3).

Table 3. Bile salt deconjugation ability and Antibiotic resistance for lactic acid bacteria.

Strain	Deconjugation of bile salts[a]				Antibiotic resistance[b]						
	TDC	GDC	TC	GC	S	C	E	AM	TE	VA	NOR
15	g	g	g	g	S	S	S	S	S	S	S
16	g	g	g	g	S	S	S	S	S	S	S
17	g	g	g	g	S	S	S	S	S	S	S
37	g	-	g	g	S	S	S	S	S	R	R
40	g	g	g	g	S	S	S	S	S	S	S
42	g	g	g	g	S	S	S	S	S	S	S
46	+	g	g	g	S	S	S	S	S	S	S
60	+	g	g	g	S	S	S	S	S	S	S
64	g	g	g	g	S	S	S	S	S	S	S
68	g	-	g	g	S	S	S	S	S	S	S
76	g	g	g	g	S	S	S	S	S	S	S
98	g	g	g	g	S	S	S	S	S	R	R
99	g	g	g	g	S	S	S	S	S	S	S
106	++	+	g	g	S	S	S	S	S	S	S
110	g	g	g	g	S	S	S	S	S	S	S
117	g	g	g	g	S	S	S	S	S	S	S
126	g	g	g	g	S	S	S	S	S	S	S
128	+	+	g	g	S	S	S	S	S	S	S
143	g	g	g	g	S	S	S	S	S	S	S
145	g	g	g	g	S	S	S	S	S	S	S
147	g	-	g	g	S	S	S	S	S	S	S
155	g	-	g	g	S	S	S	S	S	S	S
158	g	g	g	g	S	S	S	S	S	S	S
174	g	g	g	g	S	S	S	S	S	S	S

a: TC: sodium taurocholate. TDC: sodium taurodeoxycholate. GC: sodium glycocholate. GDC: sodium glicodeoxycholato; —: no growth; g: growth; +: growth and bile salt deconjugation; ++: growth and strong bile salt deconjugation **b**: : S - streptomycin (300 µg); C - chloramphenicol (30 µg); E - erythromycin (15 µg); AM - ampicillin (10 µg); TE - tetracycline (30 µg); VA - vancomycin (30 µg); NOR - norfloxacin (10 µg) S: sensible R : Resistant
Source: Elaborated by the authors.

Resistance to antibiotics is in accordance with the findings of Lee et al. (2016), in which four strains were tested. These authors found that the strains were sensitive to ampicillin, chloramphenicol, cyclohexamide, erythromycin, neomycin, streptomycin, tetracycline and rifampicin. But all strains were resistant to vancomycin at the highest amount. Nejati and Oelschlaeger (2016) also reported that all isolates were susceptible to 8 antibiotics (gentamicin, ampicillin, ciprofloxacin, erythromycin, tetracyclin, penicillin, kanamycin and nitrofurantoin) out of 9 tested (all isolates were resistant to nalidixic acid).

3.8 Selection and identification of probiotic strains

The strain selection was based on the results obtained in the tests and compared to the results found in studies by other authors. Resistance to gastrointestinal tract, antimicrobial activity against pathogens, hydrophobicity values above 40%, autoagregation levels above

75%, coaggregation with pathogens above 40%, ß-galactosidase production over 50 Miller units, BSH production, and sensitivity to antibiotics were considered for selection.

Seven LAB showed four or more of these criteria and were identified as *Lactococcus lactis* subsp. *lactis* (strain 15 and 16); *Enterococcus durans* (strain 37); and *Enterococcus faecium* (strains 106, 126, 143 and 174). There is controversy in the use of *Enterococcus* strains in food production, and therefore strains 15 and 16 were selected for the development of a functional product.

4. Conclusions

The evaluated LAB showed promising characteristics to be used as probiotic strains. The set of tests used in this study enabled selecting seven strains with desirable characteristics for any probiotic strain and as potential candidates for the formulation of new probiotic foods. Two were selected for further trials involving the possible use of this microorganism into a functional product: strain *Lactococcus lactis* subsp. *lactis* 15 showed good resistance to gastric barriers; and strain *Lactococcus lactis* subsp. *lactis* 16 was able to deconjugate bile salts, presented high percentages of autoaggregation and coaggregation, and ß-galactosidase production. Studies involving the technological potential of all strains are being performed. The strains shall be subjected to in vivo tests to compare the results.

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