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In vitro digestion as a tool for functional isolation of a probiotic potential *Lactobacillus rhamnosus*

A digestão *in vitro* como ferramenta para isolamento funcional de um potencial probiótico *Lactobacillus rhamnosus*

La digestión *in vitro* como herramienta para el aislamiento funcional de un potencial probiótico *Lactobacillus rhamnosus*

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Abstract

This study set out to isolate microrganisms strains with probiotic characteristics after simulating the *in vitro* digestion of sheep milk fermented by kefir grains. Three lactobacilli with probiotic characteristics were isolated and identified as *Lactobacillus rhamnosus*. Assays

characterized these strains as probiotic since they tolerated acid pH and bile salts, had antibiotic resistance, antagonist activity, antioxidant activity, presence of β-galactosidase enzyme and other tests revealed adhesion capacity. All strains presented antioxidant activity and survived at different pH and bile salts. These strains can be considered safe because they were susceptible to antibiotics tested, possess antagonist activity to pathogens and high β-galactosidase activity. As to adhesion criteria (hydrophobicity and autoaggregation), *L. rhamnosus* Lb16 stood out, as it also adheres to the intestinal epithelium cells of mice. The analysis of *L. rhamnosus* Lb16 can assist the dairy industry to enhance the potential human health benefits of its products. This paper is an important contribution to probiotics isolated after simulation of the *in vitro* digestion of fermented sheep milk by kefir grains, this has a differential due to its different characteristics which afforded the isolation of resistant strains to gastrointestinal conditions.

Keywords: Kefir; Sheep milk; *In vitro* digestion; *Lactobacillus rhamnosus.*

Resumo

Este estudo teve como objetivo isolar cepas de microrganismos com características probióticas após simular a digestão *in vitro* de leite de ovelha fermentado por grãos de kefir. Três lactobacilos com características probióticas foram isolados e identificados como *Lactobacillus rhamnosus*. Ensaios caracterizaram essas cepas como probióticas por tolerar pH ácido e sais biliares, apresentar resistência a antibióticos, atividade antagonista, atividade antioxidante, presença da enzima β-galactosidase e outros testes revelaram capacidade de adesão. Todas as cepas apresentaram atividade antioxidante e sobreviveram em diferentes pH e sais biliares. Essas cepas podem ser consideradas seguras porque foram suscetíveis aos antibióticos testados, possuem atividade antagonista a patógenos e alta atividade β-galactosidase. Quanto aos critérios de adesão (hidrofobicidade e autoagregação), *L. rhamnosus* Lb16 se destacou, pois também se adere às células do epitélio intestinal de camundongos. A análise de *L. rhamnosus* Lb16 pode ajudar a indústria de laticínios a aumentar os benefícios potenciais de seus produtos à saúde humana. Este trabalho é uma importante contribuição para probióticos isolados após simulação da digestão *in vitro* do leite de ovelha fermentado por grãos de kefir, este tem um diferencial devido às suas diferentes características que possibilitaram o isolamento de cepas resistentes às condições gastrointestinais.

Palavras-chave: Kefir; Leite de ovelha; Digestão *in vitro; Lactobacillus rhamnosus*.

Resumen

Este estudio tuvo como objetivo aislar cepas de microorganismos con características probióticas después de simular la digestión *in vitro* de leche de oveja fermentada por granos de kéfir. Se aislaron tres lactobacilos con características probióticas e identificaron como *Lactobacillus rhamnosus*. Los ensayos caracterizaron a estas cepas como probióticas ya que toleraron pH ácido y sales biliares, tenían resistencia a antibióticos, actividad antagonista, actividad antioxidante, presencia de enzima β-galactosidasa y otras pruebas revelaron capacidad de adhesión. Todas las cepas presentaron actividad antioxidante y sobrevivieron a diferentes pH y sales biliares. Estas cepas pueden considerarse seguras porque fueron susceptibles a los antibióticos probados, poseen actividad antagonista de patógenos y alta actividad de β-galactosidasa. En cuanto a los criterios de adhesión (hidrofobicidad y autoagregación), se destacó L. rhamnosus Lb16, que también se adhiere a las células del epitelio intestinal de ratones. El análisis de L. rhamnosus Lb16 puede ayudar a la industria láctea a mejorar los posibles beneficios para la salud humana de sus productos. Este trabajo es un importante aporte a los probióticos aislados después de la simulación de la digestión *in vitro* de leche fermentada de oveja mediante kéfir, este tiene un diferencial por sus diferentes características que permitieron el aislamiento de cepas resistentes a afecciones gastrointestinales.

Palabras clave: Kefir; Leche de oveja; Digestión *in vitro; Lactobacillus rhamnosus.*

1. Introduction

Modern consumers expect their food to be healthy. Foods that promote health over and above providing basic nutrition are defined as ‘‘functional foods”, that include foods or food ingredients that contain: probiotics, prebiotics, and symbiotic, or bioactive compounds such as antioxidants, minerals, vitamins, and active peptides (Tang et al., 2017).

Probiotics are food supplements composed of live microorganisms that benefit the host by balancing the intestinal microbiota, and fermented dairy products are one of the important sources for the transfer of beneficial microorganisms (Ghasemi-Sadabadi, et al., 2019).

Kefir is an example of functional fermented food that is characterized as being probiotic. The name is derived from the Turkish word *keyif*, meaning “good feeling” because it is said that people who drink it feel good afterward (Lima, et al., 2018).

Kefir is produced through fermentation by microorganisms lactic acid bacteria (LAB) (e.g., *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*), acetic acid bacteria (*Acetobacter*) and yeasts (e.g., *Brettanomyces*, *Issatchenkia*, *Kluyveromyces*, *Saccharomyces cerevisiae*, and *Saccharomyces exiguus*), which act in symbiosis in a complex polysaccharide (Vimercati, et al., 2020).

These form kefir grains (Lima, et al., 2017), which are small and irregular and act when inoculated into the substrate (Dertli & Con, 2017; Vimercati, et al., 2020). It depends on factors such as grain origin and storage condition, grain ratio, temperature and incubation time, final pH, grain washing, and storage (Savastano, et al., 2020; Izquierdo-González, et al., 2019).

This drink can be formulated with various types of milk: cow (Oliveira, et al., 2019), goat (Izquierdo-González, et al., 2019), or buffalo (Gul, et al., 2018). However, for such formulation, sheep milk is regarded as a high-quality product due to its short-chain fatty acids (which provide easy digestion), and it has a high amount of total solids (Lima, et al., 2018). Thus, sheep's milk fermented by kefir can be a food "carrier" of probiotics.

People who consume kefir in their diet live much longer; it is claimed to reduce lactose intolerance symptoms, stimulates the immune system, lowers cholesterol levels, has antimutagenic and anticarcinogenic properties, it contributes to balancing intestinal microbiota, and antimicrobial activity against pathogenic microorganisms (Zheng, et al., 2013).

Similarly, for these microorganisms to be viable and effective when they are inserted into the food matrix, they should also be resistant to fluids in the gastrointestinal tract (GI-tract) namely they must be able to tolerate the acid, and the action of digestive enzymes such as pepsin and pancreatic, and bile salts; and to exert their probiotic activity, sufficient numbers of bacteria need to be viable when they reach the intestine to confer a benefit to the host, the ability of microorganisms to survive and grow depends largely on their capacity to adapt to changing environments (Sumeri, et al., 2010), while the possible resistance they may have to antibiotics is their main undesirable feature. Other attributes that a probiotic must possess range from its ability to interact positively with the microorganisms in humans, to combat free radicals generated during the digestive process, to being antagonistic against pathogenic microorganisms, since they help lactose (Meira, et al., 2012).

Additionally, research on strains expressing unique characteristics that may enhance or prompt health benefits is being undertaken by characterizing naturally fermented dairy

products such as kefir. This traditional product might be an interesting source of a LAB strain with specific functional properties (Lima, et al., 2018).

Thus, this study set out to isolate microrganisms strains with probiotic characteristics after simulating the in vitro digestion of sheep milk fermented by kefir grains. The microorganisms were then evaluated for their potential probiotic potential by testing them for resistance to acid, resistance to bile, susceptibility to antibiotics, antagonism against microbial pathogens, antioxidant, enzyme β-galactosidase production and heir adhesive properties (hydrophobicity, self-aggregation, and adherence to intestinal epithelial cells).

2. Material and Methods

2.1. Fermented sheep milk

Sheep milk was purchased from a farm in the town of Vitória de Santo Antão – PE/ Brazil, Latitude: -8.11389, Longitude: -35.2915 8° 6′ 50″ Sul, 35° 17′ 29″ Oeste. The quality of the milk samples was measured at the Progene Laboratory of the University Federal Rural of Pernambuco (Brazil). The kefir grains were obtained from a private household in Pernambuco (Brazil), and the sheep milk was fermented in accordance with the method (Lima, et al., 2018).

2.2. *In vitro* digestion of fermented milk

Sheep milk fermented by kefir was used in an experiment to simulate (*in vitro*) digestion with an initial count of lactic acid bacteria of 8.5 10^8 CFU/ mL. The *in vitro* simulation of human digestion was conducted based on the method (Lima, et al., 2017), by diluting the fermented milk (kefir suspension) 1:1 in a sterile electrolyte solution, 0.6% (w/v) pepsin (Sigma-Aldrich, St. Louis, USA). Then, the pH was adjusted to pH 2.0 using different volumes of HCl solutions, to have a tight pH control. After 90 min of incubation, 1.5 mL of the solution was harvested by centrifugation and then washed twice with sterile Phosphate Buffered Saline (PBS pH 7.4) and resuspended with 1.5 mL of 1% (w/v) bile salts (Merck, Darmstadt, Germany) in PBS buffer, pH 8. The solution was finally diluted in an artificial duodenal secretion (pH 8.0) consisting of: PBS buffer, 0.3% (w/v) bile salts, and 0.1% (w/v) pancreatin (Sigma-Aldrich, St. Louis, USA). After 90 min of incubation at 37°C, a 100 µL aliquot was pipetted out and serially diluted into 0.1% peptone water solution and spread-

plate onto MRS agar (Merck, Darmstadt, Germany) supplemented with 200 mg/L of Cicloxemide (C104450 Sigma-Aldrich, St. Louis, USA) to determine the CFU/mL.

2.3. Purification and identification of lactic acid bacteria resistant to digestion

After the growth period, 24 strains, subsequently to the *in vitro* digestion procedures, were obtained from each Petri dish and cultured by the pour-plate method and incubated at 37°C for 48 hours in MRS agar with Cicloxemide (200 mg/L) to inhibit the growth of yeast. This procedure for purifying the colonies was conducted in duplicate, the colonies being subjected to Gram staining and the catalase test. Thereafter, a rod-shaped morphology was used to confirm that all colonies were Gram positive and catalase negative, and were then denominated *Lactobacillus* spp. After performing antibiotic resistance tests (data not shown), three strains with different profile results (Lb2, Lb16, and Lb24) were selected in order to conduct this study. The molecular identification of the three *Lactobacillus* spp. was then undertaken.

The molecular analysis was based on the following steps: 1) DNA Purification. A punch was used to take a 2-mm sample from cell culture stored on an FTATM Indicating Micro Card. The nucleic acids were purified using the FTA Purification Reagent (GE Healthcare, USA). 2) After DNA purification, the Polymerase Chain Reaction (PCR) of a segment of the 16S rDNA gene containing the variable regions V1 to V9 (Figure 2) was amplified. 3) The PCR products: DNA Sanger sequencing (sense and antisense) of the amplified products were purified and analyzed in agarose gel. 4) A BigDye Terminator 3 kit (Applied Biosystems, USA) was used to align sequences and to generate the consensus sequence and the fragments were sequenced on the 3730XL DNA Analyzer (Applied Biosystems, USA). 5) The generated sequences were aligned using Sequencer 5.0 software (GeneCodes, USA), to obtain the consensus sequence using the corresponding BLAST NCBI (National Center for Biotechnology Information) database. All these procedures were carried out by the company STAB VIDA (Oeiras, Portugal).

2.4. Bacterial strains and culture media

The indicator strains: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC29665, *Pseudomonas aeruginosa* ATCC27853, *Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 33019, *Staphylococcus aureus* ATCC

6538, *Salmonella enterica* serovar typhimurium ATCC 14028, *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 19117, which were held in the culture collection of the host laboratory, were used for antagonism activity. Lactobacilli cultures were grown and maintained in MRS medium (Himedia, Mumbai, India), while indicator strains were stored in brain-heart infusion medium (BHI; Himedia, Mumbai, India) as glycerol stocks at -20°C until further analysis. Prior to analysis, the cultures were propagated in their respective mediums once.

2.5. Acid and bile tolerance

The isolates were investigated for bile salt tolerance and to tolerate the acid pH, following the method (Kumar & Kumar, 2015); which showed resistance of greater than 50% at 0.3% (w/v) bile salt, were considered as bile resistant as this is the minimum threshold above which culture is considered probiotic. Isolates showing resistance greater than 50% at pH 3 were considered acid tolerant strains.

2.6. Antibiotic resistance assay

Testing the antibiotic susceptibility of the *Lactobacillus* spp. strains were performed by the disc diffusion method recommended (CLSI, 2012). Antibiotic discs used to contain cephalexin (30 µg), gentamicin (30 µg), rifampicin (5 µg), penicillin G (10 U), tetracycline (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg) and clindamycin (2 µg). All antibiotic discs were purchased from Laborclin (São Paulo, Brazil). Inhibition halos ≥ 16 mm were indicative of resistance and halos ≥ 21 mm were indicative of susceptibility to antibiotics, and all the antibiotic discs were measured in duplicate.

2.7. Antagonistic activity

The antagonistic spectrum was evaluated by the spot-on-the-lawn test (Tulini, et al., 2013), using pathogen indicator strains. Inhibition halos (<11 mm) were indicative that the lactobacilli had no antagonism; halos of (11-13 mm) show the indicator strain is of intermediary susceptibility and halos of (>13mm) show that the indicator strain is susceptible to the lactobacilli strain.

2.8. Antioxidant activity

To evaluate the antioxidant activity, a percentage reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was conducted with the protocol described (Gil-Rodríguez, et al., 2015).

2.9. β-galactosidase activity

This activity was carried according to Lima, et al., 2017 using cell-free extracts prepared by sonication. The reaction mixture comprised 50 µL of sample and 50 µL of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at a 3 mM concentration in 1 mM/ L phosphate buffer, pH 7.0. The reaction was stopped by adding 200 µL of 1 mM/ L sodium carbonate. The absorbance of the samples was measured at 405 nm on a microplate reader. A standard curve of o-nitro-phenol (ONP, Sigma-Aldrich, St. Louis, USA) was obtained using known concentrations of ONP. One enzymatic unit was defined as specific activity (U/ mg protein): 1 U is equivalent to 1 µM of ONP produced per minute.

2.10. *In vitro* cell surface hydrophobicity

The isolates were screened for their cell surface hydrophobicity using the bacterial adhesion to hydrocarbons (xylene) method by Rosenberg, et al., 1980. Isolates with an HPBI greater than 70% were arbitrarily classified as being highly hydrophobic; isolates with HPBI between 50 and 70% were classified as moderate, and isolates with HPBI lower than 50% were classified as low hydrophobic (Pringsulaka, et al., 2015).

2.11. Auto-aggregation assay

A preliminary screening of potentially adherent strains was performed to identify their auto-aggregation ability and surface hydrophobicity as described by Meira, et al. (2012) and absorbance values at 600 nm of the upper layer were measured at different time intervals (2, 4, 16 and 24 h) in a UV/Visible spectrophotometer.

2.12. *In vitro* epithelial cells adherence assay

The adhesion of the lactobacilli to the ileal epithelial cells of mice was assessed by using eight-week-old male and female Swiss albino mice (30-40 g body weight). They were fed with a conventional balanced diet (16% protein, 56% carbohydrate, 2% fat, 5.3% cellulose, and 5% vitamins and minerals) and tap water *ad libitum* to improve their adaptation. Three to four mice were housed per polycarbonate cage, with softwood chips as bedding, in an air-conditioned space at 23-25°C, air humidity of 50-60%, and under a 12 h light/dark cycle. Their intestines were collected immediately after euthanasia according to the ethical principles of animal experimentation of Brazilian College of Animal Experimentation and the prior approval of the Animal Studies Committee of the Federal University of Pernambuco (protocol number 23076.017009/2012-13).

Epithelial cells were prepared as described by Kumar & Kumar 2015. A small segment of ileum from female and male Swiss albino mice *Mus musculus*, was opened, washed twice with sterilized PBS, and incubated at 4°C for 30 min to remove the surface mucus. Epithelial cells were scraped off with the edge of a microscope slide, then suspended in PBS, and microscopically examined to ensure the removal of adherent bacteria. Further, 1 mL of each bacterial inoculum (10^8 CFU/ mL) and the epithelial cell suspension were mixed, incubated at 37°C and agitated in a shaker at 40 rpm for 30 min. The attachment of strains was studied microscopically after they had been Gram stained and after the assay had been coated with gold. Micrographs of the cells with *Lactobacillus* spp. were obtained with a Scanning electron microscope (Tescan, VEGA 3, Brno, Czech Republic).

2.13. Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze the data. Multiple comparisons were performed with Tukey's test. All statistical analyses were performed using Statistica software (version 8.0, Statsoft Inc., 2008). The statistical significance was set at $P < 0.05$ and the results are expressed as the mean ± SD (standard deviation).

3. Results

3.1. Identification of LAB digestion resistance

The effect of the successive passages through artificial saliva and the gastric phase reduced the number of colonies to 1.1 10^6 CFU/ mL, while the pancreatic phase and duodenum juices reduced the viability to 2.5 10^5 CFU/ mL. Therefore, only twenty-four *Lactobacillus* spp. candidates survived this *in vitro* model, three of which were selected for the study of their probiotic features.

After purification, evaluation of their morphological and physiological characteristics; and identification of their molecular taxonomy, were identified based on 100% identity of 16 rDNA sequences, such as *Lactobacillus rhamnosus* (Lb2, Lb16, and Lb24) when compared to database sequences (GenBank accession no. CP020464.1).

3.2. Acid and bile tolerance

The survival rates of three strains under different pH values are shown in Table 1. All strains were grown and tested at different pH, but the rates of survival at pH 2 presented a different significance. The *L. rhamnosus* Lb16 strain showed a significant ($p < 0.05$) resistance to acidic pH when compared to the other strains. At pH 3 and pH5 there was no different significance between the data, but all strains showed tolerance under pH 5.

The results of tolerance to bile salts after incubation for 24h are shown in Table 1. All tested strains survived in the presence of 0.15% bile salts. The *L. rhamnosus* Lb2 and *L. rhamnosus* Lb16 strains presented significant ($p < 0.05$) tolerance in this concentration. The *L. rhamnosus* Lb16 strain showed the best result (41.46%) when placed under stress due to 0.30% bile salts. The three *L. rhamnosus* strains tested present no tolerance at 0.45% of bile salts after incubation for 24h at 37ºC.

Table 1. Percentage of *L. rhamnosus* strains resistant to acid and bile after 24h incubation at 37°C in MRS broth.

Strains	Acid tolerance (%)[#]			Bile tolerance (%)[#]		
	pH 2	pH 3	pH 5	0.15%	0.30%	0.45%
Lb2	1.13 ± 0.01[a]	1.15 ± 0.06[a]	100.00 ± 14.88[a]	77.89 ±18.57[a]	21.23 ± 6.30[a]	0.69 ± 0.07[a]
Lb16	2.20 ± 0.06[b]	1.33 ± 0.01[a]	96.88 ± 14.21[a]	84.56 ± 14.68[a]	41.46 ± 3.50[b]	0.15 ± 0.01[b]
Lb24	1.37 ± 0.01[a]	1.22 ± 0.01[a]	98.86 ± 0.41[a]	69.00 ± 0.61[b]	21.03 ± 0.40[a]	0.96 ± 0.01[a]

[#] All the values are shown as the mean ± S.D. of three replicate experiments. [a, b] Means in the same column followed by different superscripts are significantly different ($p < 0.05$). Source: Authors.

3.3. Antibiotic resistance

The antibiotic susceptibility of all isolates was assessed by the disc diffusion method, and the results showed that the selected *L. rhamnosus* Lb2, *L. rhamnosus* Lb16, and *L. rhamnosus* Lb24 strains proved to be susceptible to all the antimicrobial agents analyzed (tetracycline, gentamicin, rifampicin, cephalexin, clindamycin, ciprofloxacin, ampicillin and penicillin), with the exception that Lb24 was not tested for clindamycin (data not show).

3.4. Antagonistic activity

The three strains of this study inhibited the growth of all microbial pathogens tested. The results are shown in Table 2. The *L. rhamnosus* Lb2, Lb16 and Lb24 strains possess similar antagonism against: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC29665, *Bacillus cereus* ATCC 33019, *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633. The *L. rhamnosus* Lb2 and Lb16 strains have antagonism against *Enterococcus faecalis* ATCC 6057 and *Listeria innocua* ATCC 33090, but the *L. rhamnosus* Lb24 strain has no activity contrary to these indicator microorganisms.

The results of antagonisms in the *Listeria monocytogenes* ATCC 19117 and *Salmonella enterica* serovar *Typhimurium* ATCC 14028 strains are significantly ($p < 0.05$) different when compared with each other, *L. rhamnosus* Lb24 and *L. rhamnosus* Lb16 presented the highest zone of inhibition against *L. monocytogenes* (37.0 ± 0.1 mm) and *S. Typhimurium* (32.0 ± 0.1 mm), respectively.

Table 2. Antagonist activity of *Lactobacillus* strains isolated after simulation of *in vitro* digestion of fermented sheep milk against various pathogens.

Indicador strains	*L. rhamnosus* with IZD[#]		
	Lb2	Lb16	Lb24
Escherichia coli ATCC 25922	26.7 ± 1.0 [a]	28.7 ± 0.4 [a]	28.0 ± 2.8 [a]
Klebsiella pneumoniae ATCC 29665	18.4 ± 3.6 [a]	22.3 ± 0.4 [a]	15.7 ± 1.7 [a]
Enterococcus faecalis ATCC 6057	18.4 ± 0.6 [a]	20.4 ± 0.2 [a]	–
Bacillus cereus ATCC 33019	16.7 ± 0.1 [a]	16.4 ± 1.0 [a]	16.3 ± 6.0 [a]
Staphylococcus aureus ATCC 6538	21.0 ± 1.3 [a]	18.7 ± 2.2 [a]	18.4 ± 1.0 [a]
Bacillus subtilis ATCC 6633	16.7 ± 3.4 [a]	16.3 ± 0.2 [a]	25.7 ± 4.4 [a]
Listeria innocua ATCC 33090	22.4 ± 3.0 [a]	22.0 ± 1.3 [a]	–
Listeria monocytogenes ATCC 19117	22.4 ± 0.2 [a]	28.4 ± 0.2 [b]	37.0 ± 0.1 [c]
Salmonella enteric serovar TyphimuriumATCC 14028	17.0 ± 0.7 [a]	32.0 ± 0.1 [c]	25.0 ± 6.7 [b]

IZD = Inhibition Zone Diameter, – = no inhibition. [#] All the values are shown as the mean (mm) ± S.D. of three replicate experiments.[a, b, c] Means in the same line followed by different superscripts are significantly different ($p < 0.05$). Source: Authors.

3.5. Antioxidant activity

All strains exhibited antioxidative activity (Table 3), the average percentages found were significantly ($p < 0.05$) different for each strain. The highest activity in this study was observed in *L. rhamnosus* Lb24 (19.15 ± 1.71%), followed by *L. rhamnosus* Lb16 (8.11 ± 0.67%) and *L. rhamnosus* Lb2 (3.91 ± 1.38%).

3.6. β-galactosidase activity

All the isolates produced β-galactosidase after 24h of incubation at 37°C (Table 3). The results of β-galactosidase activity are significantly similar between the three strains. These produced an average of 45.32 U/mg of β-galactosidase. Thus, the results of this study exhibit the potential of sheep milk fermented in kefir to add to human health as it helps humans to digest lactose.

3.7. *In vitro* cell surface hydrophobicity

The analysis of cell surface hydrophobicity of this study revealed that *Lactobacillus* strains were moderately and highly hydrophobic (Table 3). The strains with the highest hydrophobicity were *L. rhamnosus* Lb16 (94.19 ± 1.73%), *L. rhamnosus* Lb24 strain (78.06 ± 2.72%) also shows high hydrophobicity characteristics. *L. rhamnosus* Lb2 was the isolate that showed moderate hydrophobicity (54.66 ± 4.36%).

Table 3. Functional attributes of *L. rhamnosus* strains isolated after simulation of *in vitro* digestion of fermented sheep milk.

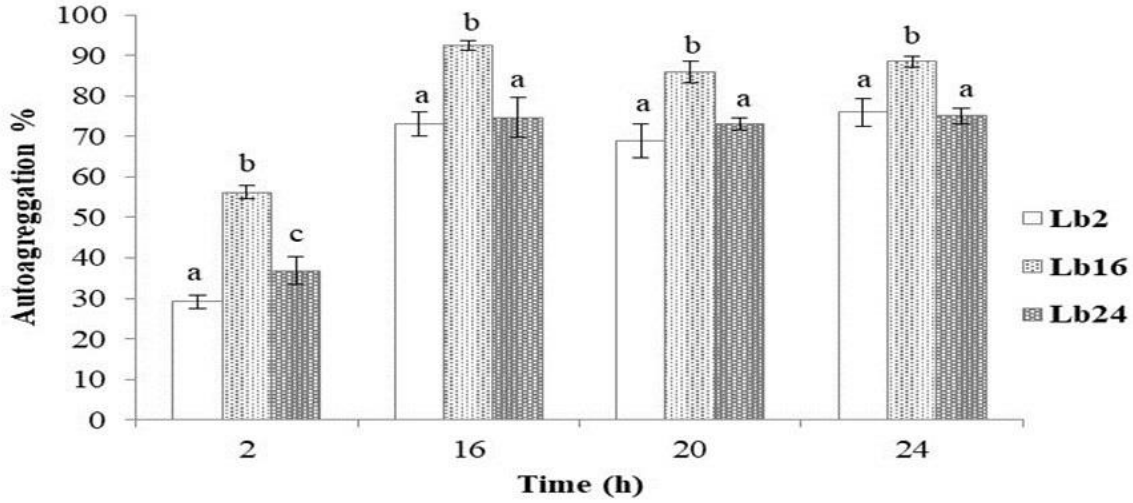
L. rhamnosus strains	DPPH[#]activity (%)	β-galactosidase[*] (U/ mg)	Hydrophobicity‡ (%)
Lb2	3.91 ± 1.38[a]	57.73 ± 18.96[a]	54.66 ± 4.36[a]
Lb16	8.11 ± 0.67[b]	39.90 ± 10.98[a]	94.19 ± 1.73[b]
Lb24	19.15 ± 1.71[c]	38.33 ± 3.86[a]	78.06 ± 2.72[c]

[#, *, ‡] All values are shown as the mean (mm) ± S.D. of three replicate experiments. [a, b, c] Means in the same column followed by different superscripts are significantly different ($p < 0.05$). Source: Authors.

3.8. Auto-aggregation assay

With regard to the auto-aggregation indices, the strains exhibited in two hours of assessment, rates that ranged between 29.19 ± 1.70% and 56.21 ± 1.58%, the latter belonging to the *L. rhamnosus* Lb16 strain. Furthermore, different times of evaluation 16h, 20h to 24h did not show significantly different results for the *L. rhamnosus* Lb2 and Lb24 strains (Figure 1). The *L. rhamnosus* Lb16 strain showed significant auto-aggregation ($p < 0.05$) at all times tested. These values were 92.41 ± 1.24% at 16h, 85.86 ± 2.69% at 20h and 88.51 ± 1.31% at 24h.

Figure 1. Results from the auto-aggregation assay of *Lactobacillus* sp. strains obtained after one *in vitro* digestion of fermented sheep milk: Lb2 (white), Lb16 (dark grey), and Lb24 (light grey). Data are expressed as mean ± SD (n = 3). Different letters (a, b, and c) represent significant differences ($P < 0.05$).

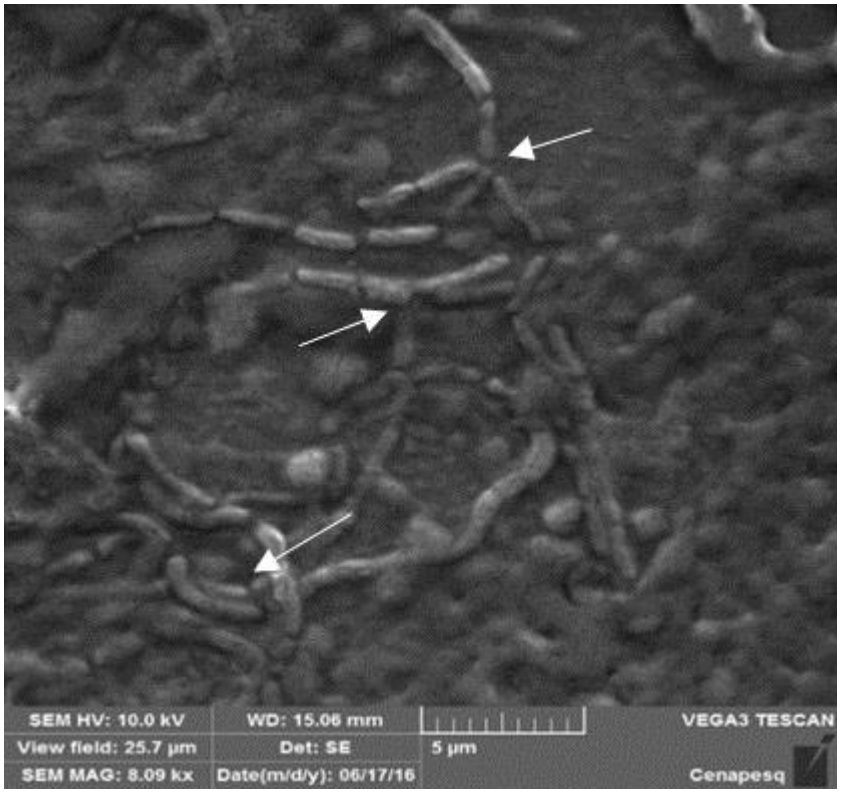


Source: Authors.

3.9. Assay of the adherence of *in vitro* epithelial cells

As mentioned by Jamaly, et al. (2011), bacteria isolates with more than 15 adhered cells per epithelial cell were considered positive in the aggregation test. Therefore, all *L. rhamnosus* strains of this study were considered positive and the results of *in vitro* adherence to epithelial cells is shown in Figure 2.

Figure 2. Results of adhesion of *Lactobacillus rhamnosus* strains obtained after one *in vitro* digestion of fermented sheep milk with scraped intestinal epithelial cells from Swiss albino mice, *L rhamnosus* Lb16 strain visualized by scanning electron microscopy. The arrow points to the site of the adhered cells.



Source: Authors.

4. Discussion

Screening for potential probiotics is the focus of the current research. Many LAB derived from fermented food were chosen because of their physiological functions. These screening criteria include the potential of the microorganism to survive and colonize the gastro-intestinal tract (GI-tract), and its ability to promote its functional and health properties (Re, et al., 2014).

In this study, sheep milk fermented by kefir grains was produced and its digestion was simulated. After the digestive process, 24 strains of *Lactobacillus spp.* were isolated (data not shown), and three strains were chosen for the study of their probiotic characteristics, and the results were compared with the existing literature. The three lactobacilli strains were identified as *Lactobacillus rhamnosus*.

The acid pH tolerance results of *L. rhamnosus* isolates were resistant at pH 5 and therefore differed from those of another research study, which show results of *L. rhamnosus* strains resistant at pH 3 above 50% (Kumar & Kumar, 2015). Strains did not hold out at low pH because they had had contact with the acid medium for 24 hours, as other studies show (Archer & Halami, 2015; Jena, et al., 2013) demonstrate that this contact has a maximum duration of 3 hours. The capacity of lactobacilli to survive at low pH remains controversial (Ren, et al., 2014).

Accordingly, as bacteria survived a simulation of the *in vitro* digestion of the fermented sheep milk by kefir and did not resist in large quantity after the pH experiment in the MRS culture medium alone, this demonstrates that, in this context, even strains that are not able to survive in low pH *in vitro* could exhibit substantial viability when consumed together with milk (Tuo, et al., 2013).

This study has shown that the *L. rhamnosus* strains have tolerance to simulated gastric juice. Our results are in accordance with these reports and suggest that the isolates persist and subsequently pass through the GI-tract.

Tolerance to gastric stress is one of the primary prerequisites of probiotic functionality as ingested strains need to survive the hostile environment of the GI-tract. The human liver secretes nearly a liter of bile into the small intestine in a total concentration that ranges between 0.3–0.5 % on average. Therefore, the presence of bile in the intestine affects the viability of probiotic bacteria. Accordingly, (Vinderola & Reinheimer, 2003), considered that LAB strains are bile resistant only when they show resistance of ≥ 50% at 0.3% (w/v) bile salt. In this context, only the *L. rhamnosus* Lb16 strain possesses moderate resistant to bile salts. Most studies so far have shown that most of the strains survived well under such conditions (Archer & Halami, 2015; Tuo, et al., 2013), which suggests a potential recuperation of the initial levels while they pass through the small intestine.

Investigating susceptibility to antibiotics is a common evaluation feature of safe probiotic bacteria (Kumar & Kumar, 2015). In our study, none of the selected strains were resistant to the antibiotics tested and these strains are considered safe. Other studies have found that *Lactobacillus* strains from a dairy origin are resistant to antibiotics (Zheng, et al., 2013; Kumar & Kumar, 2015). However, these studies justify resistance to antibiotics, as an intrinsic, non-transferable resistance. Safe probiotics did not have the resistant genes, because these could antagonize colonization in the mucosal sites of the intestines and to produce lactic acid and bacteriocin to inhibit the proliferation of pathogens.

According to Santos, et al. (2003), the lactobacilli are used in the prevention and treatment of gastrointestinal disorders, such microorganisms act in two different ways: with the production of antimicrobial substances and by competitive inhibition of enteropathogen attachment to epithelial cells. In the present study, the antagonist activity of all isolates inhibited microbial pathogens. Other studies also evaluated the antimicrobial activity of lactobacilli strains of dairy origin (Kumar & Kumar, 2015), from animals (Jena, et al., 2013) and humans (Ren, et al., 2014), but they did not observe strains with such a large spectrum of activity (greater than or equal to six indicator strains) as that in this study. So, it can be determined that the source of *L. rhamnosus* isolation is directly related to its activity. Thus, it can be concluded that the antagonistic ability of strains derived from sheep milk fermented by kefir is higher than that of those from other sources.

Natural antioxidants that protect the body from free radical damage and reduce the incidence of chronic diseases, some *Lactobacillus* possess antioxidant abilities and are considered successful probiotics (Tang, et al., 2017).

All *Lactobacillus* strains of this study exhibited antioxidant activity. The results showed an average of 10.39%. Ren et al., 2014 found antioxidant activity by capturing the hydroxyl radical ranging between 10.37% and 94.26% from *Lactobacillus* isolated from fermented foods and the human intestine. Depending on the production of enzymes or compounds on the surface of cells, these microbial cells may exhibit different natures of antioxidant activity.

Lactose maldigestion or intolerance may be treated using bacteria from fermented milk products that contain the lactose hydrolyzing enzyme β-galactosidase, it is produced by most lactobacilli with both hydrolase and transglycosylase activities, which is advantageous from the technological and health points of view (Meira, et al., 2012). This enzyme is intracellular which seems to act, when it is launched, by breaking the bacterial cells during transit of the intestine, so the intracellular extract was used in the present study. All *L. rhamnosus* of the present study showed the β-galactosidase enzyme. This enzyme has also been found in other studies that used *Lactobacillus* strains isolated from fermented products, dairy, and feces (Meira, et al., 2012; Jena, et al., 2013). The β-galactosidase presence has gained importance for potential applications such as for probiotic cultures in the dairy industry or as producers of galacto-oligosaccharides, prebiotic ingredients.

Hydrophobicity is a very important criterion that enables probiotics to bind and reside in the intestines of the host for a long time, and they also confer a competitive advantage for bacterial maintenance in the GI-tract. A higher hydrophobicity is shown when microbial cell

surfaces presented (glyco-) proteinaceous material on the cell surface (Kos, et al., 2003). Such material could be potential mediators in the initial steps involved in adhesion. It was found in this study 94.19 % from *L. rhamnosus* Lb16. This value is higher than that of other studies that used *Lactobacillus* strains and xylene for analysis: 70.9% (22), 88.0 % (Meira, et al., 2012). According to Pringsulaka, et al. (2015), the *L. rhamnosus* Lb24 strain (78.06 ± 2.72%) also shows high hydrophobicity characteristics.

Aggregation between microorganisms of the same strain may achieve an adequate mass to form biofilms or adhere to the mucosal surfaces of the host and thus is of considerable importance in several ecological niches. The auto-aggregation from *Lactobacillus* strains of the present study followed the same behavior of the lactobacilli studied by Meira, et al. (2012), namely, values increased throughout the incubation time and the results were more apparent from 16 h until the final incubation period. However, our results were higher for *L. rhamnosus* Lb16 (84.39%) than the 79.8% found by Meira, et al. (2012) at 24h of assay. According to the considerations of Ren, et al. (2014), these *Lactobacillus* strains possess a great chance of adhesion in epithelial cells.

As suggested by the results of hydrophobicity and auto-aggregation, all strains of this study showed adhesiveness to the intestinal cells of mice. These results were considered positive according to the considerations of Jamaly, et al. (2011), who mention that the adhesion efficiency of at least 15 bacteria per epithelial cell. In other studies, the adhesiveness of *Lactobacillus* strains was considered positive when an evaluation was made of the epithelial cells obtained from mice (Kumar & Kumar, 2015), and by cell culture type Caco-2 (Kotzamanidis, et al., 2010). Adhesion is a prerequisite for colonization, Kotzamanidis, et al. (2010) mention that the ability of *Lactobacillus* to adhere to the epithelial cells of the host was suggested to be beneficial for probiotics because this transient intimate association is administered to exchange mediators between the bacterium and the host immune system and the bacterium competes with enteric pathogens for binding sites.

Studies with probiotic evaluation of *Lactobacillus* isolated from kefir have already been conducted (Zheng, et al., 2013). However, this paper is an important contribution to the study of probiotics isolated after simulation of the *in vitro* digestion of fermented sheep milk by kefir grains. This fermented milk has a differential due to its different characteristics which afforded the isolation of resistant strains and other gastrointestinal conditions of probiotic characteristics.

5. Final Considerations

Three *Lactobacillus rhamnosus* strains were isolated from sheep milk fermented by kefir grains after *in vitro* digestion process and these strains show appropriate properties for probiotic application. The *L. rhamnosus* Lb16 strain stands out from the others because it showed global characteristics that stood out front of the other strains (e.g., antagonist activity). This study indicates that the *L. rhamnosus* Lb16 strain analyzed can be useful in the production of dairy foods for potential human health benefits.

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