



EVALUATION OF *LACTOBACILLUS PARACASEI* LP11 AND *LACTOBACILLUS RHAMNOSUS* 64 POTENTIAL AS CANDIDATES FOR USE AS PROBIOTICS IN FUNCTIONAL FOODS

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ABSTRACT

Desirable characteristics for probiotic microorganisms were evaluated in *Lactobacillus rhamnosus* 64 and *Lactobacillus paracasei* LP11. Both strains were tested for resistance to lysozyme and simulated gastrointestinal fluids, determination of cell wall hydrophobicity, evaluation of antibiotic susceptibility, antagonist activity against pathogens and the ability to use prebiotics. The safety determination was performed by an in vivo test, where the occurrence of bacterial translocation to liver was evaluated after daily oral administration of the strains for 10 days to mice. All results were promising, with the exception of resistance to simulated gastrointestinal fluids, that was unsatisfactory, with a decrease in viable cell concentrations of up to 5.08 log CFU/mL. The hydrophobicity profile of the strains ranged between 8.47 and 19.19%. Both showed satisfactory resistance to lysozyme, with survival rates above 80%, strains were able to antagonize *Escherichia coli* V517, *Salmonella enteritidis* OMS-Ca, *Staphylococcus aureus* 76 and *Listeria monocytogenes* ATCC 15313, and displayed satisfactory use of lactulose, inulin and P95 raftilose as prebiotics. No bacterial translocation was observed after the administration of the strains to mice. The results obtained herein justify the use of *L. rhamnosus* 64 and *L. paracasei* LP11 in new investigations for the future application of these microorganisms as probiotics.

Keywords: Lactobacilli; Safety; Resistance to gastrointestinal fluids; Hydrophobicity; prebiotics

INTRODUCTION

Functional foods currently represent a significant portion of the food market (Siró, Kápolna, Kápolna, & Lugasi, 2008). It is believed that the growth of the functional food market is related to increasing population awareness about the close relationship between food and health (Lähteenmäki, 2013). Functional foods present bioactive components in their composition capable of optimizing the metabolic and/or physiological functioning of the organism, resulting in disease prevention, especially chronic non-degenerative diseases (Gul, Singh, & Jabeen, 2016; Tur & Bibiloni, 2016). Among components that give food functional classifications are antioxidant vitamins, phytosterol, omega 3, fiber, prebiotics and probiotics (Dias, Botrel, Fernandes, & Borges, 2017; Schieber, 2012).

Probiotics are living microorganisms that, when administered in suitable amounts, confer benefits to host health (FAO/WHO, 2002). The main genera used as probiotics are *Lactobacillus* and *Bifidobacterium* (Sharma & Devi, 2014). When ingested in sufficient amounts, probiotics present benefits through a variety of mechanisms, including decreases in the number of pathogenic microorganisms through competitive exclusion (competition for binding sites and nutrients) and bacteriocin production (Niamah, 2010; Hegarty *et al.*, 2016), stimulation of the immune system (Ashraf & Shah, 2014; Castillo, de Moreno de LeBlanc, Galdeano, & Perdigón, 2012), vitamin synthesis (LeBlanc *et al.*, 2011), production of short chain fatty acids (acetate, propionate and butyrate) through fiber fermentation (Gibson, 2004), and lactose digestion, reducing the effects of lactose intolerance (Staudacher, 2015).

Currently, probiotic-containing foods represent an important share of the functional food market (Begum *et al.*, 2017), with emphasis on the dairy industry (Granato, Branco, Cruz, Faria, & Shah, 2010; Vijaya Kumar, Vijayendra, & Reddy, 2015). The development of these foods involves several challenges. The

microorganism must be capable of surviving the adverse conditions inherent to food product processing and storage (Champagne, Gardner, & Roy, 2005). However, before planning the development of probiotic-containing food, challenges involving the proper selection of these microorganisms must be overcome. A series of criteria involving safety, functional and technological characteristics must be met for a microorganism to be considered probiotic (Mattila-Sandholm *et al.*, 2002).

Probiotic selection begins with the precise identification of the microorganism at the genus, species and strain levels. It is recommended that the microorganism be of human origin, due to the greater propensity to adapt to the human intestine, also considering, the greater possibility of adhesion to intestinal epithelial cells (FAO/WHO, 2002; Shewale, Sawale, Khedkar, & Singh, 2014). The microorganism must be completely safe for human ingestion, with a non-pathogenic history and no antibiotic resistance gene transfer capabilities (FAO/WHO, 2002; Havenaar, Brink, & Huis In 't Veld, 1992). In addition, the probiotic candidate must be able to withstand the adverse conditions of the gastrointestinal tract, including contact with gastric acid and bile acids in the stomach and intestine, respectively (Tuomola, Crittenden, Playne, Isolauri, & Salminen, 2001). It is important that the microorganism display the ability to colonize the human intestine, albeit transiently, through adhesion to the intestinal epithelium (FAO/WHO, 2002; Shewale, Sawale, Khedkar, & Singh, 2014). Finally, probiotics are expected to exhibit antimicrobial activity against potentially pathogenic bacteria, resistance to antibiotics and be capable of stimulating the host's immune system (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). After fulfilling these criteria, *in vivo* tests are applied to determine the possible beneficial effects on the maintenance or recovery of human health.

Considering that microorganism characterization is indispensable for the adequate development of functional foods, the aim of the present study was to evaluate the potential of *Lactobacillus paracasei* LP11 and *Lactobacillus rhamnosus* 64 as probiotic candidates by *in vitro* and *in vivo* tests.

MATERIAL AND METHODS

Microorganisms

Frozen culture of commercial probiotic (*Lactobacillus paracasei* LP11) was obtained from local provider. *Lactobacillus rhamnosus* 64 isolated from newborn human feces (Vinderola *et al.*, 2008), were evaluated herein. Before each analysis, freeze-dried *L. paracasei* LP11 and *L. rhamnosus* 64 frozen in MRS broth (de Man, Rogosa and Sharpe) were activated in MRS broth (Biokar, Beauvais, France) during 16 h at 37 °C under aerobic conditions. The identity of frozen culture of commercial probiotic manufacturer has been kept undisclosed for confidentiality reasons.

Resistance to lysozyme

The lysozyme resistance analysis was performed as described by Vizoso Pinto, *et al.* (2006) and Zago *et al.* (2011) with modifications. Microorganisms grown overnight in 10 ml of MRS broth at 37°C were centrifuged, washed twice and resuspended in 2 ml of phosphate buffer (0.1 M, pH 7.0). Subsequently, 10% of the bacterial suspension was inoculated into a sterile electrolytic solution (SEE) (SEE; 0.22 g/l CaCl₂, 6.2 g/l NaCl, 2.2 g/l KCl, 1.2 g/l NaHCO₃) in the presence of 100 mg/l lysozyme (Sigma-Aldrich). The control comprised the bacterial suspension inoculated into the SEE without lysozyme. Survival rates were calculated as a percentage of CFU/ml after 30 and 120 min compared to the counts determined at time zero.

Resistance to simulated gastrointestinal fluids

The *in vitro* evaluation of the resistance of free microorganisms to simulated gastric fluid (SGF) and simulated intestinal fluids (SIF) was performed according to the method described by Gbassi *et al.* (2009). The SGF was composed of 9 g/L sodium chloride (Synth, Diadema, Brazil) and 3 g/l pig stomach pepsin (Sigma-Aldrich, St. Louis, USA) in distilled water with pH adjusted to 1.8 with hydrochloric acid. The SIF was prepared with 9 g/L sodium chloride (Synth, Diadema, Brazil), 10 g/L pancreatin, 9 g/L bovine trypsin (Sigma-Aldrich, St. Louis, MO, USA), and 3 g/L of bile salts (Oxgall, Difco, Hampshire, UK) in distilled water and pH adjusted to 6.5 with sodium hydroxide. The microorganisms were inoculated into the SGF and maintained at 37°C under agitation. Viable cell counts were performed in MRS at 0, 60 and 120 minutes. Subsequently the remaining SGF analysis material was inoculated into the SIF, proceeding with appropriate pH control and adjustment and viable cell counts in MRS after 0, 90 and 180 minutes.

Hydrophobicity

Strains were grown in 10 mL of MRS broth for 16 h at 37 °C. Cultures were centrifuged (6,000 rpm, 6 min) and washed twice with phosphate buffer (0.1 M, pH 7.0). The cultures were then suspended in the same buffer, and the optical density (OD) adjusted to approximately 1.0 at 560 nm. Subsequently, 0.6 ml of η-hexadecane (Sigma-Aldrich, St. Louis, USA) were added to 3.0 ml of the bacterial suspension and, after homogenization for 2 minutes by vortexing, the mixtures were incubated at 37 °C, for 1 hour, for phase separation. The lower aqueous phases were carefully removed with a sterile Pasteur pipette and absorbances (A1) were recorded at 560 nm following the method described by Vinderola and Reinheimer (2003) and Duary *et al.* (2011). The following formula was applied to determine the results, expressed as percentage of bacterial adhesion to the solvent:

$$H\% = (1 - A1/A0) \times 100 \quad (1)$$

Where A0 is the initial adjusted absorbance and A1 is the absorbance of the lower aqueous phase resulting of the η-hexadecane addition to the bacterial suspension.

Antibiotic susceptibility

The two strains were submitted to a sensitivity analysis for eight antibiotics (Laboclin, Pinhais, Brazil): cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), erythromycin (15 µg), neomycin (30 µg), penicillin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). Susceptibility was determined according to the Kirby & Bauer method described in Bauer, Kirby, Sherris, & Turck (1966). A 10⁸ CFU/mL suspension of each strain was seeded on plates containing MRS agar. Subsequently, paper disks embedded with each antibiotic were placed on the surface of the culture medium and the plates were then incubated for 48 h at 37 °C. After incubation, the inhibitory halos formed on each disk were measured

with the aid of a pachymeter. The results were qualitatively analyzed, and the microorganisms were classified as sensitive, moderately sensitive or resistant to the evaluated antibiotic.

In vitro antagonism

The agar spot method was adopted to evaluate the *in vitro* antagonistic activity of the two lactobacilli strains (Schillinger & Lücke, 1987). Four pathogens (10⁷ CFU/ml) were used, namely *Escherichia coli* V517, *Salmonella enteritidis* OMS-Ca, *Staphylococcus aureus* 76 and *Listeria monocytogenes* ATCC 15313. Additionally, the ability of the neutralized cell-free supernatant to promote an antagonistic effect was assessed according to the methodology proposed by Uhlman, Schillinger, Rupnow, & Holzapfel (1992) and detailed by Vizoso Pinto *et al.*, (2006). Briefly, the supernatants were obtained from the overnight culture of *L. paracasei* LP11 and *L. rhamnosus* 64 in MRS broth maintained at 37 °C in aerobic conditions. After culturing, the cultures were centrifuged at 7,200 × g for 10 minutes, neutralized with a sterile 5 M NaOH solution and the supernatants were boiled for 5 min to inactivate viable cells.

Prebiotic use

The capacity to use inulin, polydextrose, fructooligosaccharides and lactulose was evaluated, according to Zago *et al.* (2011). Glucose (Sigma-Aldrich) was used as a positive control. Initially, all prebiotics and glucose were solubilized in distilled water and sterilized by filtration. The strains were inoculated (1.5% inoculum; 10⁶ CFU/ml) into test tubes containing 3 ml of modified MRS broth, which contained 2% of each of the four prebiotics or glucose as the sole carbon source. The tubes were incubated at 37 °C for 24 h under aerobic conditions. The use of prebiotics and glucose was evaluated by measuring the optical density of each culture at 560 nm (OD560). The cell growth rate in the presence of each prebiotic was calculated according to the following formula:

$$\text{Growth rate} = [(MRS_{Sp} - MRS_{Sb}) * 100] / (MRS_{Sg} - MRS_{Sb}) \quad (2)$$

Where MRS_{Sp} is the MRS containing each prebiotic, MRS_{Sb} is the MRS without a carbon source (negative control) and MRS_{Sg} is MRS containing glucose (positive control).

In vivo safety evaluation

Animals management and microorganism administration procedures

BALB/c male mice, five to six weeks old, were provided by the Centro de Medicina Comparada, Instituto de Ciencias Veterinarias (ICiVet-Litoral), Faculty of Veterinary Sciences of the National University of Litoral (Esperanza, Santa Fe, Argentina). Prior to the beginning of the study, the project was submitted for evaluation and approved by the National University of Litoral Ethics and Research Committee. Twelve animals were used in the study. Animals were kept for 1 week in the animal facility at the Instituto de Lactología industrial (UNL-CONICET) for acclimatization, at 21 ± 1 °C, in 12h/12h light/dark cycle, with total air renewal rates of 20 volumes/h and *ad libitum* access to a standard rodent diet (Cooperación, Buenos Aires, Argentina) and sterile tap water.

Bacterial translocation assay

The mice were divided into 3 groups, each group comprising 4 animals. For 10 consecutive days, mice received 0.3 ml/day of a cell suspension of *L. rhamnosus* 64 (group 1), *L. paracasei* LP11 (group 2) or a sterile saline solution (control group). The cell suspension contained 10⁷ CFU/mL of lactobacilli. After 10 days of administration, mice were anesthetized with a ketamine, xylazine and acepromazine cocktail and sacrificed by cervical dislocation.

Liver and spleen were aseptically removed, homogenized in PBS and plated (1 mL) on MacConkey agar (Biokar, Beauvais, France) according to Vinderola *et al.* (2005). Plates were incubated at 37 °C for 48 h. Results were expressed as positive (presence of bacterial colonies on the plates) or negative (absence of bacterial colonies on the plates, no translocation).

Statistical analyses

Statistical analyses were performed in a fully randomized design and two replicates. Data were analyzed by analysis of variance (ANOVA) and Tukey Test using the Statistical Analysis System (SAS, 1995, version 9.1.3). The results were considered statistically significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Lysozyme resistance

The two evaluated strains presented satisfactory results regarding lysozyme resistance, with *L. paracasei* LP11 displaying higher resistance than *L.*

rhamnosus 64 ($p < 0.05$), as shown in Figure 1. After exposure for 120 minutes to the electrolytic solution at a 100 mg/l lysozyme dilution simulating *in vivo* saliva conditions, *L. rhamnosus* 64 showed a 1.8 log CFU/g decrease, while *L. paracasei* LP11 showed a 0.83 log CFU/g decrease.

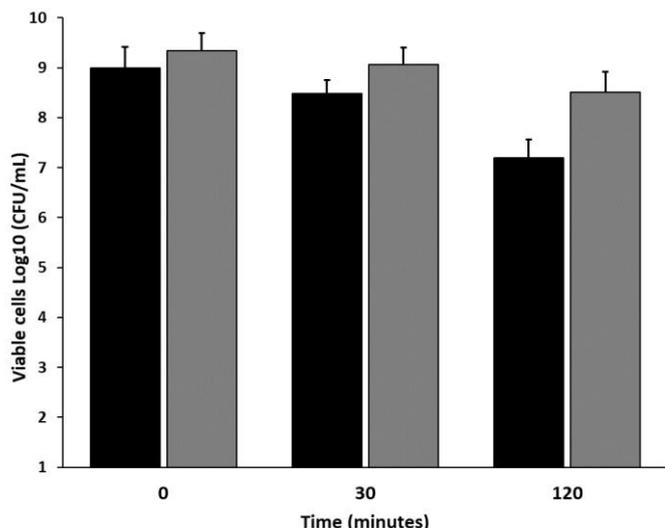


Figure 1 Survival of *L. paracasei* LP11 (■) e *L. rhamnosus* 64 (■) in CFU / mL after exposure for 120 minutes to the electrolytic solution containing 100 mg / l lysozyme.

Other authors have reported values close to those reported herein. Vizoso Pinto *et al.* (2006) also evaluated the resistance of *L. johnsonii* LA1 to lysozyme, exposing the microorganism for 60 minutes to a solution containing lysozyme at a similar concentration and detected a decrease of one logarithmic cycle in relation to the initial microorganism concentration. Zago *et al.* (2011) evaluated the lysozyme susceptibility of 27 *L. plantarum* strains isolated from cheeses. The results varied between 3.24 and 99.97% survival rates after 120 minutes of exposure to the solution containing 100 mg/l of lysozyme, and 15 strains showed survival above 68%, a result considered satisfactory by the authors. Köll *et al.* (2008) evaluated the sensitivity of 22 lactobacilli strains specifically selected as probiotic candidates for oral health performance, and observed after determining the presence of growth inhibition zones that all strains were resistant to lysozyme at concentrations ranging from 0.2 to 10 mg/ml.

Recently, some researchers have suggested the use of probiotics for oral health promotion (Bizzini *et al.*, 2012; Haukioja, 2010), thus encouraging research to develop vehicles for probiotic delivery into the oral cavity. Some of the research published so far (Heinemann, Carvalho, & Favaro-Trindade, 2013; Saha, Tomaro-Duchesneau, Daoud, Tabrizian, & Prakash, 2013) propose the incorporation of probiotic bacteria in oral disintegration films, allowing the probiotics to comprise the transient microbiota of the buccal mucosa and teeth, permitting protective action against pathogenic microorganisms that promote dental diseases. For this type of study, probiotic sensitivity to lysozyme is crucial, since this enzyme has been shown to be effective in killing several types of Gram-positive bacteria by promoting cell wall rupture and subsequent cell lysis (Köll *et al.*, 2008).

As observed in several studies, including the one conducted by Zago *et al.* (2011), lactobacilli sensitivity to lysozyme is a strain-dependent trait. When incorporated into a food product, the sensitivity of the microorganism to lysozyme is not as worrying, since exposure of the probiotic to the enzyme is very short. As seen herein, during the first 30 minutes, the impact of lysozyme on *L. paracasei* LP11 and *L. rhamnosus* 64 concentrations was very low. However, it is always prudent to assess such sensitivity, considering that there may be a deleterious summation of buccal secretion and gastrointestinal secretions.

Resistance to simulated gastrointestinal fluids

In order for the ingested probiotics to reach the intestinal lumen, their main site of action, they must be able to withstand adverse gastrointestinal (GIT). In order to have a physiological impact, it is desirable that probiotics reach the GIT in concentrations higher than 7 log CFU/g (Gill & Rutherford, 2001; Ya *et al.*, 2008).

After exposure to gastrointestinal fluids, the behavior of *L. paracasei* LP11 and *L. rhamnosus* 64 was observed after 120 minutes exposure to simulated gastric fluid ($p < 0.05$). While *L. rhamnosus* 64 showed a 1.31 log CFU/g decrease, the decrease of *L. paracasei* LP11 concentrations reached 3.37 log CFU/g (Fig. 2). However, the behavior of both strains in relation to exposure to simulated intestinal fluids for 90 minutes was not significantly different ($p > 0.05$), with no significant concentration decreases. After gastrointestinal fluid exposure, data

indicated that *L. rhamnosus* 64 presented a final concentration of 6.05 log CFU/g, while *L. paracasei* LP11 presented a final concentration of 3.92 log CFU/g.

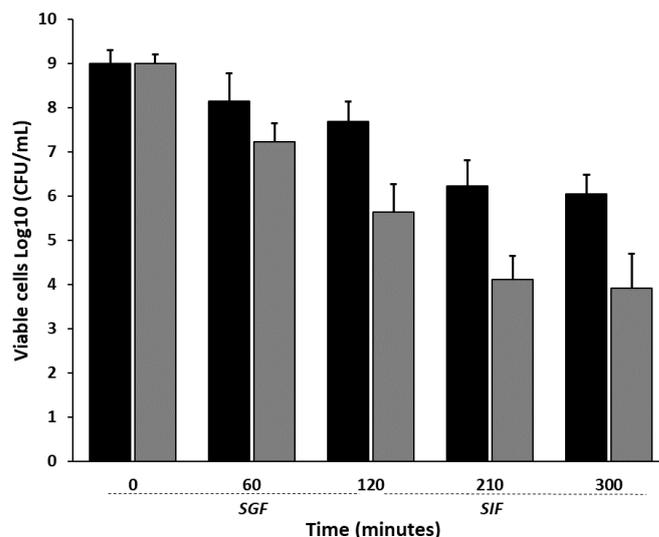


Figure 2 Survival of *L. paracasei* LP11 (■) e *L. rhamnosus* 64 (■) in CFU / mL after 300 min exposure to simulated gastrointestinal fluids (SGF), with 120 minutes of exposure in simulated gastric fluid and 180 minutes of exposure in simulated intestinal fluids (SIF).

Significant decreases in the concentrations of viable lactobacilli cells exposed to gastrointestinal fluids have been reported by several authors. Ilha, da Silva, Lorenz, de Oliveira Rocha, & Sant'Anna (2015) characterizing *L. paracasei*, reported a 4.25 log CFU/mL decrease after exposure to pH 2.0 for 3 hours. Ding & Shah (2007) evaluated the resistance of *L. rhamnosus* and *L. paracasei* at pH 2.0 for 2 hours and reported a decrease of 6.69 and 6.61 log CFU/mL for each microorganism, respectively. Pimentel-González, Campos-Montiel, Lobato-Calleros, Pedroza-Islas, & Vernon-Carter (2009) reported that *L. rhamnosus* when exposed under simulated gastric conditions at pH 2.3 for 2 hours presented a 1.87 log CFU/mL decrease in relation to initial concentrations.

Some probiotic-candidate microorganisms may present high sensitivity to gastrointestinal fluids, but promising results in other characterization tests or when included in a food matrix. One way to overcome this high sensitivity is the use of microencapsulation technology, which has demonstrated satisfactory efficiency in increasing survival against GIT conditions (Bosnea *et al.*, 2014; Cook *et al.*, 2012; de Vos *et al.*, 2010; Pedroso *et al.*, 2012). Several microencapsulation techniques have been successfully applied in the protection of probiotic cells, with emphasis on spray chilling (Okuro, de Matos, & Favaro-Trindade, 2013; Pedroso, Dogenski, Thomazini, Heinemann, & Favaro-Trindade, 2013) and complex coacervation (Bosnea *et al.*, 2014; Oliveira *et al.*, 2007). In this context, although *L. paracasei* LP11 and *L. rhamnosus* 64 showed high sensitivity to gastrointestinal fluids, the use of the microencapsulation technology could be advantageous, depending on the results of the other probiotic potential characterization tests.

Hydrophobicity

Significant attention has been paid to cell wall hydrophobicity profiles in the characterization of microorganisms with probiotic potential. Although hydrophobicity is not the only factor related to the complex adhesion process of microorganisms to the intestinal epithelium, some authors state that the initial stage of this fixation is mediated by physico-chemical interactions that include hydrophobicity and chemical charges (Barbosa, Silva, Martins, & Nicoli, 2005; Pelletier *et al.*, 1997). It is believed that the higher the hydrophobicity of the surface of the microorganism, the greater its adhesion capacity (Kos *et al.*, 2003; Rosenberg, Gutnick, & Rosenberg, 1980). Although some studies claim that lactobacilli generally present a hydrophobic cell surface (García-Cayueta *et al.*, 2014), it is possible to find studies with varied hydrophobicity values, including values below 10% (Dhewa, Bajpai, Saxena, Pant, & Mishra, 2010; Zago *et al.*, 2011) and above 90% (Mangoni *et al.*, 2011; Van Coillie *et al.*, 2007).

In the present study *L. rhamnosus* 64 presented 19.19% hydrophobicity, while *L. paracasei* LP11 presented 8.47%, within the range reported in the literature. Other studies that have also evaluated *L. rhamnosus* and *L. paracasei* strains support the claim that cell surface hydrophobicity profile, as well as several other characteristics expected for probiotic microorganisms, is strain-dependent. Pelletier *et al.* (1997) cites values between 8.3 and 9.6% and 8.7 and 26.5% hydrophobicity for *L. paracasei* and *L. Rhamnosus* strains, respectively, while Xu, Jeong, Lee, & Ahn (2009) reported hydrophobicity values close to 25 and 46% for *L. paracasei* and *L. rhamnosus* GG, respectively. Polak-Berecka, Waško, Paduch, Skrzypek, & Sroka-Bartnicka (2014) also used hexadecane as solvent to

evaluate the hydrophobicity of *L. rhamnosus* and reported values between 11 and 16%.

It is important to note that, although it is desirable for a probiotic to have a hydrophobic outer surface, so that it is able to withstand the effects of intestinal peristalsis of the intestinal fluid stream and not be eliminated, this should not be a limiting feature in probiotic selection, since, as observed by Santos *et al.* (2016), probiotics with remarkable positive functional characteristics and low hydrophobicity are also available.

Antibiotic susceptibility analysis

The results of the antibiotic susceptibility analysis of the two evaluated lactobacilli strains are summarized in Table 1. *L. rhamnosus* 64 showed

sensitivity to five of the eight evaluated antibiotics, presenting moderate sensitivity to penicillin and tetracycline and resistance to erythromycin. On the other hand, *L. paracasei* LP11 showed sensitivity to penicillin and moderate sensitivity to erythromycin and tetracycline. Both strains were resistant to vancomycin, which corroborates several studies, both recent (Aazami *et al.*, 2014; Haghshenas *et al.*, 2015; Santos *et al.*, 2016) and otherwise (Hamilton-Miller & Shah, 1998). In summary, the resistance profile of *L. rhamnosus* 64 and *L. paracasei* LP11 to the evaluated antibiotics did not differ compared to reports published in the literature (Gueimonde, Sánchez, G. de los Reyes-Gavilán, & Margolles, 2013; Köll *et al.*, 2008; Santos *et al.*, 2016; M. Sharma & Devi, 2014; Vizoso Pinto *et al.*, 2006).

Table 1 Antibiotic resistance profile of *L. rhamnosus* 64 and *L. paracasei* LP11

| Strains | Antibiotics | | | | | | | |
|--------------------------|-------------|------|------|-----|-----|-----|-----|-----|
| | Cefe | Cefo | Ceft | Eri | Neo | Pen | Tet | Van |
| <i>L. rhamnosus</i> 64 | S | S | S | R | S | MS | MS | R |
| <i>L. paracasei</i> LP11 | S | S | S | MS | S | S | MS | R |

Antibiotics: Cefepime (Cefe) (30 µg), cefoxitin (Cefo) (30 µg), ceftriaxone (Ceft) (30 µg), erythromycin (Eri) (15 µg), neomycin (Neo) (30 µg), penicillin (Pen) (10 µg), tetracycline (Tet) (30 µg) and vancomycin (Van) (30 µg). S: sensitive, MS: moderately sensitive, R: resistant.

Probiotic resistance to antibacterial drugs does not necessarily motivate concerns; on the contrary, the use of resistant probiotics may be useful for the restoration of the intestinal microbiota after treatment with antibiotics (Gueimonde *et al.*, 2013). However, a problem arises when the resistance is transferable, allowing pathogenic microorganisms to acquire resistance through bacteria used as probiotics (Bernardeau, Vernoux, Henri-Dubernet, & Guéguen, 2008; P. Sharma, Tomar, Goswami, Sangwan, & Singh, 2014). The resistance of lactobacilli strains to erythromycin and vancomycin detected herein has been reported in the literature as an intrinsic resistance without the risk of horizontal transfer (Ammor, Belén Flórez, & Mayo, 2007; Gad, Abdel-Hamid, & Farag, 2014; Rabia & Shah, 2011).

In vitro antagonism

L. paracasei LP11 and *L. rhamnosus* 64 were able to promote an antagonistic effect against the four evaluated pathogens (*Escherichia coli* V517, *Salmonella*

enteritidis OMS-Ca, *Staphylococcus aureus* 76 and *Listeria monocytogenes* ATCC 15313). A pathogen growth inhibition zone greater than 6 mm was observed in all spots containing active lactobacilli cells (Table 2). No positive inhibition results regarding antagonist activity of the supernatants obtained from the growth of the lactobacillus strains neutralized with 5 M NaOH and boiled for 5 minutes was detected, indicating that the inhibition observed by active cells was not due to the production of thermoresistant bacteriocins. Similar reports have been published by other authors evaluating lactobacilli strains (Maragkoudakis *et al.*, 2006; Poppi *et al.*, 2015; Vizoso Pinto *et al.*, 2006). Those studies suggest that the antagonistic effect in this case is possibly due to the production of organic acids. This statement, however, is not unanimous in the literature. Santos *et al.*, (2016), for example, reported that neutralized supernatants from lactobacillus isolates isolated from cocoa fermentation were able to inhibit *E. coli* ATCC 25922 growth, noting that *L. plantarum* supernatants also inhibited *S. enterica* var. Typhimurium ATCC 14028 and *Shigella flexi* ATCC11060.

Table 2 Evaluation of *in vitro* antagonism capacity of *L. rhamnosus* 64 and *L. paracasei* LP11

| Strains | Pathogens | | | |
|--------------------------|-----------|----|----|----|
| | EC | SE | AS | LM |
| <i>L. rhamnosus</i> 64 | + | + | + | + |
| <i>L. paracasei</i> LP11 | + | + | + | + |

Microorganisms: *Escherichia coli* V517 (EC); *Salmonella enteritidis* OMS-Ca (SE); *Staphylococcus aureus* 76 (AS); *Listeria monocytogenes* ATCC 15313 (MO). Presence of inhibition halo (+).

Prebiotic use

Considering that prebiotics are non-digestible food ingredients capable of selectively stimulating probiotic growth (Gibson & Roberfroid, 1995), the evaluation of the probiotic microorganism capacity to use prebiotics is indispensable, especially when their use in symbiotic products is envisaged. Considering that recent research has demonstrated that the ability to use prebiotics is a strain-dependent trait (Mandadžieva, Ignatova-Ivanova, Kambarev, Iliev, & Ivanova, 2011; Zago *et al.*, 2011), the two lactobacilli strains studied herein were evaluated for their capacity to use four prebiotics: inulin, polydextrose, P95 rafhiosis and lactulose. As displayed in Figure 3, *L. paracasei* LP11 showed high growth in the presence of inulin and fructooligosaccharides, with cell growth rates of 75% and 86%, respectively, in relation to the control. However, this strain presented growth rates lower than 35% in the presence of polydextrose or lactulose. *L. rhamnosus* 64, on the other hand, presented high growth only in the presence of lactulose, of 91% in relation to the control, and cellular growth rate of less than 40% in the presence of the other evaluated prebiotics. When the growth performance of the two strains was evaluated in the presence of each prebiotic, inverse behaviors (p <0.05) were observed, except for polydextrose, which did not result in any growth difference between the two strains (p > 0.05).

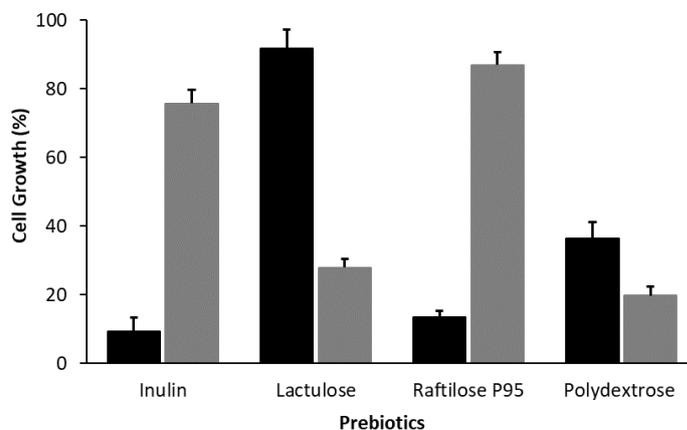


Figure 3 Use of inulin, lactulose, P95 rafhiosis and polydextrose by *L. paracasei* LP11 (■) and *L. rhamnosus* 64 (■) during growth. The Prebiotic Utilization Capacity (CUP) values were expressed as percentage of cell growth in relation to glucose (100% of use), used as control after 24 hours of incubation.

Lactulose that, in this study, promoted a high growth rate for *L. rhamnosus* 64 and low for *L. paracasei* LP11 was also the target of a study performed by Zago *et al.* (2011) regarding its use by 27 *L. plantarum* strains isolated from cheese. The authors reported that lactulose, among seven evaluated prebiotics, provided the highest cell growth rate for all the investigated strains, with values close to 100% in relation to the control. Kunová, Rada, Lisova, Ročková, & Vlková (2011) also reported successful results regarding the use of lactulose by several lactobacilli species, although inulin promoted the highest growth rates. Watson *et al.*, (2013) observed satisfactory growth rates for 21 lactobacillus species when using lactulose, noting that the same strains were not able to use inulin and polydextrose.

The studies available in the literature present mixed results, reinforcing the assertion that probiotic use of prebiotics is a strain-dependent characteristic (Tropcheva *et al.*, 2013), and prevent any kind of generalization. However, a frequently reported behavior is the low capacity or inability of lactobacilli to use polydextrose (Mäkeläinen, Saarinen, Stowell, Rautonen, & Ouwehand, 2010; Watson *et al.*, 2013; Zago *et al.*, 2011), a fact corroborated by the findings reported herein, for both *L. rhamnosus* 64 and *L. paracasei* LP11. Polydextrose presents a complex structure, and is difficult and slow to degrade, requiring the performance of several intestinal microbiota microorganisms to promote at least partial molecular degradation (Röytiö & Ouwehand, 2014). Although results from *in vitro* tests with isolated probiotic microorganisms are not successful in terms of the ability to use polydextrose, it is important to note that some clinical studies have confirmed the ability of this prebiotic to beneficially modulate intestinal microbiota (Costabile *et al.*, 2012; Hooda *et al.*, 2012; Jie *et al.*, 2000; Lamichhane *et al.*, 2014).

Inulin and fructooligosaccharides are among the most commonly used prebiotic ingredients for the development of symbiotic foods (Burić, Cardarelli, Filisetti, & Saad, 2007; Fornelli *et al.*, 2014; Moayednia, 2014; Paula K Okuro, Thomazini, Balieiro, Liberal, & Fávoro-Trindade, 2013; Paz, Oliveira, Kairuz, & Ramón, 2014), although the ability of these probiotics to use these probiotic microorganisms is not always tested. With recognized bifidogenic effects (Búriço, Fagundes, Trindade, & Vasconcelos, 2007; Shoaib *et al.*, 2016), inulin and fructooligosaccharides have also demonstrated positive effects for lactobacilli. Souza Oliveira, Perego, de Oliveira e Converte (2012) reported satisfactory results regarding the effect of inulin on growth and metabolism of *L. rhamnosus* in co-culture with *Streptococcus thermophilus*. Makras, Van Acker e De Vuyst (2005) observed that *Lactobacillus paracasei* subsp. *paracasei* 8700: 2 isolated from breast milk was able to use inulin and fructooligosaccharides. On the other hand, in the studies conducted by Adebola, Corcoran and Morgan (2014), *L. acidophilus* NCTC 1723, *L. delbrueckii* ssp. *bulgaricus* NCTC 12712 and *L. brevis* NCIMB 11973 were not able to use inulin as a carbon source.

It is pertinent to point out that the fact that a prebiotic does not stimulate the growth of the probiotic present in the symbiotic food does not mean that its activity is useless, since other strains belonging to the intestinal microbiota can be stimulated, promoting host benefits. However, it is strongly expected that the prebiotic should display the ability to stimulate the growth of the accompanying probiotic in a symbiotic food.

In vivo safety evaluation

No bacterial translocation to liver or spleen was observed for the dose of *L. paracasei* LP11 and *L. rhamnosus* 64 assessed, suggesting that these microorganisms did not alter the integrity of the intestinal epithelium or the balance of the resident microbiota.

The translocation of bacteria from the enteric microbiota to extraintestinal sites due to microorganism administration is one of the most worrisome adverse effects of this practice. Considered a probiotic toxicity factor, bacterial translocation may be related to the pathogenesis process of opportunistic strains (Zago *et al.*, 2011). Several authors have investigated the translocation possibility of probiotic-candidate lactobacilli strains. Medici, Vinderola, & Perdígón (2004) reported negative results for translocation when evaluating *L. acidophilus* and *L. paracasei* isolated from cheese. Similar results were reported by Zago *et al.* (2011) when evaluating the administration of *L. plantarum* strains derived from cheeses.

In 2005, a study carried out by Vinderola *et al.* (2005) suggested that the possibility of bacterial translocation could be related to the dosage. When studying the immunomodulatory effect of kefir in mice, the authors verified the occurrence of bacterial translocation to spleen and liver when daily doses of kefir diluted at 1/10 and 1/50 ratios were administered. However, when the kefir was administered at 1/100 and 1/200 ratios, bacterial translocation was negative. Gregoret, Perezlindo, Vinderola, Reinheimer, & Binetti (2013), who administered doses higher than the one evaluated herein, did not verify bacterial translocation at daily doses of 2×10^8 CFU for up to 7 days for *L. paracasei*, *L. rhamnosus* and *L. gasseri* isolated from newborn feces. As the risk of bacterial translocation may correlate with dosage, it is important to perform oral ingestion safety tests as a preliminary step in food development.

CONCLUSIONS

The *in vitro* evaluation performed herein revealed that the two lactobacilli strains presented satisfactory results in most of the conducted tests, demonstrating potential for use as probiotic microorganisms in functional foods. The analysis of resistance to gastrointestinal conditions was the only feature that fell short of the desired. However, such resistance can be improved by microencapsulation technology, which has shown promising results. In the safety test performed *in vivo*, the ingestion of both strains was shown to be safe at the dosage administered in an animal model. Once resistance to gastrointestinal conditions has been improved, the microorganisms must undergo *in vivo* tests in order to determine their possible functionalities in the organism, to be subsequently applied in a food matrix.

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