# Probiotic Characterization And Safety Assessment of *Lactococcus Lactis* Subsp. *Lactis* R7 Isolated From Ricotta Cheese

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# Abstract

The aim of this study was to identify and characterize *in vitro Lactococcus lactis* R7 isolated from commercial ricotta cheese. The results from phenotypic characterization demonstrated that *L. lactis* R7 had growth potential in a wide temperature range (15 °C and 45 °C), ability to tolerate high osmotic concentrations (sodium chloride 4.0 %), ability to growth in acidic and alkaline condition (pH 2.0 and 9.6), and ability to sugar fermentation (glucose, maltose and ribose). The findings confirm that *L. lactis* R7 belong to the genus *Lactococcus*. The results from molecular identification by 16S RNA identified the isolate as *Lactococcus lactis* subsp. *lactis* R7. The phenotypic characteristics combined with the molecular identification, indicate that the isolate R7 belongs to the *lactis* subspecies. The isolate *L. lactis* R7 was tolerant to acidity and bile salts. In the intestinal tract, cell concentrations were higher than 7.98 log CFU.mL$^{-1}$ in the presence and absence of bile salts. *L. lactis* R7 showed antioxidant and inhibitory capacity for lipid peroxidation. It also demonstrated capacity for self-aggregation (25.8%), coaggregation (18.3%) and hydrophobicity (11.1%). The antagonist activity of the isolate was greater against *Staphylococcus aureus* (12.2 mm), when compared to *Escherichia coli* (11.1 mm) and *Salmonella enteritidis* (9.5 mm). In the MTT assays, *L. lactis* R7 did not show cytotoxicity to VERO cells at the evaluated concentrations. In conclusion, *L. lactis* R7 isolated from ricotta cheese presented probiotic characteristics and compatible safety aspects for use as a food technology culture.


# Introduction

The increased need for a healthier lifestyle and the development of products with functional characteristics have led to a growth in the demand for probiotic foods (Peres et al. 2012). As a result, interest in the search for new bacterial isolates has also increased, mainly of lactic acid bacteria (LAB). The purpose is to obtain microorganisms well adapted to the intrinsic and extrinsic conditions of the food, some of them with technological and/or probiotic potential (Huang et al. 2017).

Lactic acid bacteria are the main group of microorganisms used in fermentation processes in the food industry. In addition, these bacteria are also widely used for their probiotic properties and consist of a variety of genera, including, but not limited to, *Lactobacillus, Lactococcus, Streptococcus, Leuconostoc, Pediococcus* and *Enterococcus* (Ross et al. 2017).

Thei isolation and characterization of the LAB strains is important, since they can be advantageous for obtaining lineages with different taxonomic, technological and functional characteristics (Ortu et al. 2007). These new strains should not present virulence and antimicrobial resistance factors, ensuring safe food (Kondrotiene et al. 2020).

Even though there are currently well-characterized and available probiotic strains, the isolation and characterization of new strains have great relevance, considering that each strain can bring specific health benefits. In addition, studies demonstrate bactericidal and/or probiotic potential of microbiota consisting of LAB (Hermanns et al. 2014; Jeronymo-Ceneviva et al. 2014).

*Lactococcus lactis* is the main starter culture, used worldwide in the production of various fermented dairy products, including cheeses, fermented milks and butter, due to its high capacity for proteolysis and acidification. They are categorized as homofermentative bacteria, with formation of lactic acid as the main product, in addition to having the capacity to produce nisin (Cavanagh et al. 2015). However, the demand of consumers for products with diverse flavors and the appeal to healthiness cause the expansion of innovative products containing bacteria with desired characteristics (Manini et al. 2016). In this way, the aim of this study was to identify and characterize *in vitro L. lactis* R7 isolated from commercial ricotta cheese, regarding probiotic potential, antioxidant capacity, safety aspects and cytotoxicity.

# Materials And Methods

### *Lactococcus lactis* subsp. *lactis* R7

The isolate *L. lactis* R7, obtained from commercial ricotta cheese, was grown on selective medium — De Man, Rogosa and Sharpe (MRS) agar (Merck, USA) — and kept at -70 °C in MRS broth with 30% glycerol.

### Preliminary identification

The isolate was stained using the Gram technique and visualized under an optical microscope, for observation of morphology. Additionally, a loop containing a liquid culture of each isolated bacterium was tested for the expression of the catalase enzyme, using 3% hydrogen peroxide ($H_2O_2$) (Celiberto et al. 2018), and the ability to ferment glucose with gas ($CO_2$) production, using *L. fermentum* ATCC 9338 (positive control) and *L. plantarum* ATCC 8014 (negative control) (Santos et al. 2016). The tubes that showed turbidity of medium and gas production were classified as heterofermentative, while those only with turbidity of medium were classified as homofermentative.

### Phenotypic characterization

For phenotypic characterization it was analyzed the tolerance of *L. lactis* R7 to pH, temperature, sodium chloride (NaCl) and the ability to ferments carbohydrates, especially maltose. The pH, temperature and sodium chloride concentration was analyzed as described to Menconi et al. (2014) with some modification. The rationale for these two points was mainly based on food matter passage time through the gastrointestinal tract of human.  An overnight culture of *L. lactis* R7 was used as the inoculum, with the cells being centrifuged and resuspended in 0.9% sterile saline. The suspension (100 µL) was inoculated into 10 mL of MRS broth in each test tube. Two incubation time points (2 h and 4 h) were evaluated for each of the variables (pH, temperature and NaCl). It was analyzed the tested for survivability using two different pHs (2.0 and 9.6), as well as it was performed the temperatures tested 20 °C and 45 °C, and the NaCl concentrations tested were 3.5 and 6.5% (w/v). Fermentation test for glucose, maltose, and ribose was determined by acid formation in accordance to Tanigawa et al. (2010). Overnight *L. lactis* R7 (4 mL) at 37°C for 24 h were pelleted by centrifugation at 2.000 x *g* for 20 min, and the cell pellet was suspended in 2 mL of sterile saline (0.9%). Bacterial suspension (130 µL) was

inoculated into the test medium (modified MRS broth, with the glucose replaced by 2% of the test substrate) supplemented with 0.005% chlorophenol red as the pH indicator, and then incubated at 26 °C for 1, 4, and 7 days. The acid production in 4 mL of broth was determined by the color change in the medium from red (pH 6.8) to yellow (pH 5.4).

### Extraction of genomic DNA and molecular and genotypic analysis

### Extraction of genomic DNA from *L. lactis* R7 isolate

The extraction of genomic DNA was performed by the chemical lysis method, in which the *L. lactis* R7 isolate was first inoculated into Petri dishes containing BHI agar. These were incubated at 37 °C for 24 h to evaluate the purity of the culture. Genomic DNA was extracted from the isolate into 1.5 mL microtubes containing 100 µL of EAR Buffer and 5 µL of Proteinase K (20 $mg.mL^{-1}$ Invitrogen®) at 55 °C for 4 h. After cooling the samples at room temperature, 750 µL TE Buffer was added. Then, after centrifugation, 1 µL of the supernatant containing the extracted DNA (25 ng) was used for the polymerase chain reaction (PCR) technique.

### Amplification of DNA by polymerase chain reaction

The 16S rRNA gene of the *L. lactis* R7 isolate was amplified using bacterial universal primers corresponding to *E. coli* positions 27F (5'-AGATTTGATCMTGGCTCAG-3) and 1492R (5'-TACGGYTACCTTGTTACGAC TT-3) (Lane 1991). The PCR reaction mixture consisted of 12.5 µL of PCR Master Mix (Promega, Madison, WI, USA), genomic DNA template 25 ng (0.5 µL), primer 27F (2.5 µL=12.5 pmol), and primer 1492R (2.5 µL=12.5 pmol), made up to a final volume of 25 µL with nuclease-free water. The 16S rRNA gene was amplified using a 35-cycle PCR: initial denaturation, 95 °C for 5 min; subsequent denaturation, 95 °C for 0.5 min; annealing temperature, 50 °C for 1 min; extension temperature, 72 °C for 1 min; and final extension, 72 °C for 5 min.

### Phylogenetic analysis

DNA polymerase-mediated amplification of templates was carried out in the presence of mixtures of dNTPs, fluorescently labeled dideoxynucleotide triphosphates, and primer 519r (5-WATTACCGCGGCKGCTG-3), using a Sanger (BigDye) terminator kit (Applied Biosystems). Extension products were fractionated by capillary electrophoresis, using an Applied Biosystems 3730-XL DNA sequencing machine and following the manufacturer's instructions. BioEdit (Hall 1999) was used to edit sequences, and a 16S-based phylogenetic tree was inferred from ClustalX alignments, using the neighbor joining method (Saitou and Nei 1987) in MEGA version 5.0 (Tamura et al. 2011). Genetic distance was calculated based on the Kimura two-parameter model of nucleotide evolution (Kimura 1980). The support of nodes was assessed with 1,000 bootstrap replications.

### Antioxidant capacity

The methods used for assessing antioxidant capacity were the thiobarbituric acid reactive substance (TBARS) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, as described by Pieniz et al. (2014).

### Probiotic characterization of *L. lactis* R7

Tolerances to low pH and the presence of bile salts were tested by determination of bacterial viability, after exposition of the isolate to solutions with low pH values: 2.0, 3.0, 4.0, 5.0 and 7.0; and in final concentrations of bile salts of 0.1, 0.25, 0.5, 1.0 and 1.5% (w/v) Oxgall (Difco™, USA) solutions. Both methodologies were carried out according to Perelmuter et al. (2008).

### Survival in simulated gastrointestinal tract

*Lactococcus lactis* R7 was evaluated for its survival during 15, 30, 60, 120, 180 and 240 min of exposure to simulated gastric fluids and 60, 120, 180 and 240 min to simulated intestinal fluids. The test was performed according to Huang et al. (2004), with modifications. A 0.2 mL aliquot of bacterial suspension was inoculated into 1.0 mL of saline solution (control) and of simulated gastric or intestinal fluids. Simulated gastric fluids consisted of 3 mg of pepsin dissolved in 1 mL of NaCl solution (0.5%), acidified with HCl to pH 2.0 and 3.0. Simulated intestinal fluids consisted of 1 mg of pancreatin and 1 mL of NaCl solution (0.5%), adjusted to pH 8.0, with or without 1.5% of bile salts (1:1 mixture of sodium cholate and sodium deoxycholate). Both solutions were sterilized by filtering through 0.22 µm membranes (Millipore, Bedford, USA). Viable cell counts were performed with serial dilution and plating on MRS agar, incubated at 37 °C for 48 h under anaerobic conditions.

### Auto-aggregation, coaggregation and hydrophobicity assays

Auto-aggregation and co-aggregation assays were carried out *in vitro,* according to Collado et al. (2008), and both were expressed as percentage values. *Listeria monocytogenes* was used for the co-aggregation assay.

The test for bacterial adhesion to hydrocarbons was carried out according to Vinderola et al. (2003). To evaluate bacterial adhesion to the hydrocarbon, toluene reagent (Synth®) was used. The bacterial suspension (3 mL) with absorbance of 0.25 ± 0.02 was agitated in a Vortex® for 1 min with 400 µL toluene. After 2, 4 and 24 h at 37 °C, the aqueous phase was carefully removed and the absorbance (600 nm) was read in an ultraviolet-visible (UV-Vis) spectrophotometry. The result was expressed as the mean of the percentage values ± standard deviations.

### Antagonist activity of *L. lactis* R7

Antibacterial activity was verified using the Kirb-Bauer disk-diffusion test developed by Bauer (1959). Inhibition zones around the LAB isolates represented positive results, expressed in millimeters. The pathogens used were *E. coli* ATCC 8739, *S. enteritidis* ATCC 13076, *L. monocytogenes* ATCC 19114 and *S. aureus* ATCC 25923.

## Safety aspects

### Deoxyribonuclease (DNase) test

DNase activity was tested as described by Bannerman (2003), using the medium DNase Test agar with toluidine blue (Himedia, São Paulo, Brazil). The appearance of clear zones around the bacterial colonies indicated DNase positive results. A clear halo around the colonies was also indicative of a positive result. The positive control used was *S. aureus* ATCC 25923.

### Hemolytic activity

The hemolytic activity was performed according to Foulquié Moreno et al. (2003). *Lactococcus lactis* R7 was tested for hemolytic activity using blood agar (7% v/v horse blood), being incubated for 48 h at 37 °C. The results were interpreted as follows: if there are green-hued zones around the colonies (α-hemolysis) or there is no effect on the blood agar plates (γ-hemolysis), isolates are considered non-hemolytic; however, if they show blood lyse zones around the colonies, they are classified as hemolytic (β-hemolysis).

### Gelatinase activity

The detection of gelatinase production was performed according to Marra et al. (2007). This test used a negative control (BHI and 12% gelatin) and a positive control (*S. aureus* ATCC 25923). Solidification of the medium indicates negative result and liquefaction indicates positive result.

### Lipase production

Lipase production was performed according to Barbosa et al. (2010), with modifications. Lipolytic activity was identified by the formation of opaque halos around the colonies.

### Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed according to standard disk diffusion method, recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2017). Six antimicrobials commonly used in the hospital environment were tested: clindamycin-2 (CLI), chloramphenicol-30 (CLO), meropen-10 (MER), erythromycin-15 (ERI), vancomycin-30 (VAN) and penicillin-10 (PEN). The diameter of inhibition zones was measured after incubation for 24 h at 35 °C. The isolate was classified according to the CLSI criteria: as susceptible, intermediate or resistant to the antimicrobial (CLSI 2008). Data were expressed in millimeters (mm).

### Evaluation of biofilm formation ability

This assay was performed in a 96-well microtiter plate, according to the method described by Stepanović et al. (2000). *Staphylococcus epidermidis* ATCC 25923 was used as a positive control, and a negative control was performed with BHI alone.

### Cytoxicity assay (MTT)

The MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out according to Vaucher et al. (2010) method. In brief, VERO cell cultures ($3.10^5$ cells.$mL^{-1}$) were prepared in 96-well plates; then, 200 mL of serial dilutions (0.02 to 2.5 mg.$ml^{-1}$) of bacterial suspension of *L. lactis* R7, or 20 mg.$mL^{-1}$ SDS (positive control) were added. Fresh E-MEM was used as negative control. The plates were incubated for 24 h at 37 °C in a humidified 5% $CO_2$ atmosphere. Later, the medium was removed from all wells by suction, and 50 mL of 1 mg.$mL^{-1}$ MTT solution (USB Corporation) prepared in E-MEM medium was added to each well. The plates were incubated for 4 h at 37 °C. The MTT solution was removed without disturbing the cells, and 100 mL of DMSO were added to each well for dissolving formazan crystals. The plates were gently shaken for 5 min, and crystals were completely dissolved. Afterwards, the absorbance was read on a 680 Microplate Absorbance Reader (Bio-Rad Laboratories) at 540 nm.

### Statistical analysis

Data was analyzed using two-way analysis of variance (Two-way ANOVA) and Tukey test at 5% significance level for comparison of means, using Graphpad Prism 8.0 (GraphPad, La Jolla, CA, USA). The analyses were performed in triplicate, and the results were calculated with the means of two independent experiments.

# Results And Discussion

### Phenotypic and molecular characterization of the *L. lactis* R7 isolate

The isolate *L. lactis* R7 showed typical characteristics of the LAB group: Gram-positive, catalase-negative and homofermentative. Therefore, it produced lactic acid as the final product of carbohydrate fermentation, avoiding flavor and undesirable characteristics in the final product. Many species of the *Lactococcus* has been widely used in the food industry, since present microbiological safety. Mostly, showed probiotic characteristics, contributing sensorially to products; they also increase the nutritive value of products through the synthesis of vitamins, proteins and essential amino acids (Giraffa 2004). As well, it is important the use of LAB in the control of pathogens through the production of metabolites with antimicrobial properties, as well as in food preservation (Ayala et al. 2019).

For the identification of isolate, it was performed phenotypic characterization (Table 1). The results obtained demonstrated that *L. lactis* R7 had growth potential in a wide temperature range (between 15 °C and 45 °C). The bacterial capability to grow at high temperature is a good characteristic, as it could be interpreted as indicating an increased rate of growth and lactic acid production. Moreover, a high fermentation temperature decreases contamination by other microorganisms (Ibourahema et al. 2008; Menconi et al. 2014). *L. lactis* R7 demonstrated also ability to tolerate high osmotic concentrations of sodium chloride at 4.0 %, and this is a characteristics of the *L. lactis* subsp. *lactis*. This result was in accordance with others studies showing that sodium chloride tolerance might be strain-dependent

(Bevilacqua et al. 2010). The knowledge about sodium chloride tolerance of an *L. lactis* isolate allows a rational selection and control of starter cultures in the manufacture of dairy products, especially cheese, with different levels of salt. Thus, incorporation of strains more sensitive to salt in initial cultures would potentially increase autolysis and the release of intracellular enzymes, neutralizing with sensory defects, such as bitterness, often described in low-salt cheeses (Kristensen et al. 2020).

The isolate showed ability to growth in acidic and alkaline condition (pH 2.0 and 9.6). One of the most important criteria in selecting bacterial with probiotic potential is that they are tolerant to acidic conditions. For ensure that the viability and functionality to be satisfactorily exercised, probiotic bacteria need to survive at pH 1.5 to 2.0, this condition resembles the passage in the host's human stomach (Dunne et al. 2001). This characteristic will ensure survival in this habitat, together with the other probiotic characteristics described below. In the same way, *L. lactis* R7 showed ability to sugar fermentation: glucose, maltose and ribose. In accordance to other authors (Desmasures et al. 1998; Fernández et al. 2011) these results are important parameters because *L. lactis* subsp. *lactis* is distinct from *L. lactis* subsp. *cremoris* according to five phenotypic criteria: the ability to grow at 40 °C, in 4.0 % of NaCl, pH 9.2, the ability to ferment maltose and the ability to deaminate arginine, for all of which *L. lactis* subsp. *cremoris* strains are reported as negative. Furthermore, Drici et al. (2010) reported also that the ablitlity to resist at pH 9.6, at 45 °C of temperature, but not in the presence of 6.0% NaCl, differentiates between the *Enterococcus* and *Lactococcus* gender, because *Lactococcus* does not tolerate a concentration of NaCl higher than 4%. Thus, the findings of the present study confirm that the isolated *L. lactis* R7 belong to the genus *Lactococcus*.

The currently, *L. lactis* subspecies defined are *lactis*; *cremoris*; *hordniae* and *structae*. The strains of the subspecies *lactis* and *cremoris* are central components of the culture blends used in the commercial production of cheese, which, through lactic acid, influence the flavor, texture and quality of the final products. However, the heat sensitivity of *cremoris* subspecies compared with *lactis* subspecies often precludes their use in certain applications. The taxonomic classification of *L. lactis* subsp. *lactis* and subsp. *cremoris* is based on phenotype and differentiated on the basis of growth temperature, salt tolerance and arginine utilization. However, with progress in molecular methods in the last decade, it has become clear that comparison of strains from a broad range of different environmental niches challenge these phenotypic distinctions and that a combination of genotype and phenotype is required to describe strains of this species (Pérez et al. 2011; McAuliffe 2018).

For this reason, the isolate *L. lactis* R7 was analyzed by 16S rRNA gene. The results from molecularly identification showed that *L. lactis* R7 as belonging to the genus *Lactococcus*, species *lactis*, subspecies *lactis* (*L. lactis* subsp. *lactis*), which was deposited in GenBank under accession number KF879126, showing similarity of 99.8% with other sequences, based on the 16S rRNA gene, previously deposited in GenBank (Figure 1). The phenotypic characteristics showed in the present study combined with the molecular identification, indicate that the isolate R7 belongs to the *lactis* subspecies.

### Probiotic characterization of the *L. lactis* R7 isolate

The ability to tolerate acidity has been shown by some authors to be strain-dependent (Manini et al. 2016; Flach et al. 2018; Cruxen et al. 2019). The isolate was evaluated for its ability to survive at different pH values (2.0, 3.0 and 4.0), as shown in Figure 2(A). As can be seen, *L. lactis* R7 showed cell viability of 7.08 log CFU.mL$^{-1}$, after 4 h of cultivation at pH 2.0. At pH 3.0 and 4.0, the log reduction was 1.31 and 1.20 log CFU.mL$^{-1}$, respectively, at the end of 4 h of exposure, indicating acidity tolerance. Guo et al. (2009) reported that the variation in acidity tolerance may be related to the difference in H+-ATPase activity, which controls the intracellular concentration of H+, maintaining pH homeostasis and cell viability (Meira et al. 2012).

Bile, even in low concentrations, can inhibit the growth of microorganisms, and survival in the concentration of 0.3% has been considered a critical value for the screening of isolates resistant to this compound (Manini et al. 2016). *Lactococcus lactis* R7 was able to maintain cell viability (7.15 log CFU.mL$^{-1}$) after 4 h of exposure to bile salts at a concentration of 0.5%, as seen in Figure 2(B).

The viability of *L. lactis* R7 to the simulated gastrointestinal tract was evaluated in the presence and absence of food (milk). The results are shown in Figure 3(A). The cellular concentration of *L. lactis* R7 was reduced in the presence of pepsin (pH 2.0) over time, with a logarithmic reduction of 2.73 cycles ($p < 0.05$). On the other hand, when the isolate was exposed to simulated gastric fluid and the food matrix, the cell reduction was significantly lower (1.64 log CFU.mL$^{-1}$), demonstrating that the food provided protection to the microorganism ($p < 0.05$).

Studies report that milk proteins have technological properties, such as buffering capacity, good emulsification and ability to form networks even at low concentrations, ensuring good survival of microorganisms during digestion (Ranadheera et al. 2012; Prasanna et al. 2018). Similar results were found by Hwanhlem et al. (2010), who reported that *Lactobacillus* strains were able to maintain their viability when exposed to acidity conditions with pH between 2.5–4.0; however, at lower pH values there was a reduction in their viability. Pieniz et al. (2014) evaluated the *E. durans* LAB18s isolate from fresh Minas cheese, which showed high survival capacity in the presence of simulated gastric fluids containing pepsin (pH 3.0) and simulated intestinal fluids containing pancreatin (pH 8, with or without addition of bile salts). The viability of *E. durans* LAB18s was also satisfactory when exposed to pH 3.0 and 4.0, although a decrease in viable cell counts was observed when pH 2 was evaluated, a result that is similar to that found in the present study.

In the simulated intestinal tract (Figure 3(B)), cell concentrations above 7.98 log CFU.mL$^{-1}$ were obtained, both in the presence and in the absence of bile salts (0.5%), at all times. The exception was the time of 4 h in the presence of bile salts, where there was a significant reduction of 2.86 logarithmic cycles, reaching the minimum value so that the isolate can have beneficial effects as a probiotic (6 log CFU.mL$^{-1}$). As observed in the results of bile salt tolerance (Figure 3(B)) and in the literature reports, *L. lactis* R7 showed activity of the bile salt hydrolase (BSH) enzyme in the small intestine. This is because the resistance to bile salts of some isolates is related to the activity of the enzyme that helps hydrolyze conjugated bile,

reducing its toxicity (Ilha et al. 2015; Chen et al. 2017). These results show that this isolate has resistance to bile salts and suggest the possibility of its survival in the human gastrointestinal tract.

The probiotic potential of LAB isolated from milk and sheep cheeses was evaluated by Meira et al. (2012), who found that these bacteria were able to tolerate low concentrations of bile salts (0.1 and 0.3%). Only the LCN 56 strain (*L. plantarum*) presented cell concentrations above the limit of detection when exposed to 0.5% of bile salts. In addition, none of the evaluated strains presented viability above the limit of detection after the 4 hours of incubation. Considering that the concentration of bile salts of 0.15 to 0.3% has been recommended for the *in vitro* evaluation of the passage through the small intestine (Huang and Adams 2004), the isolate *L. lactis* R7 showed probiotic potential under the studied conditions.

The survival of ingested probiotics faces stomach acidity and the presence of bile salts in the duodenum as the main obstacle (Chu-Ky et al. 2014), this resistance to acids also remains in acidic food applications. However, brief and mild exposure to acids can result in greater acid resistance in future exposures (Mills et al. 2011; den Besten et al. 2013).

In addition, to the probiotic potential, *L. lactis* R7 also showed anticarcinogenic potential in colorectal cancer induced in animal models, in a previous study of this research group (Jaskulski et al. 2020). There are no reports so far in the literature about such effects presented by this isolate. These results are promising because, in addition to demonstrating the potential for colorectal cancer stabilization, they challenge science to explore the effects of *L. lactis* R7 and continue research.

### Auto-aggregation, co-aggregation and hydrophobicity

The auto-aggregation capacity of the *L. lactis* R7 isolate increased exponentially over the evaluated time, achieving a maximum value at 4 h (25.8 ± 0.03%) of incubation and then decreasing. The same profile was verified in relation to the coaggregation capacity (18.3 ± 0.02%) of the isolate. It is known that auto-aggregation and co-aggregation among bacteria play an important role in preventing the colonization of surfaces of the intestinal mucosa by pathogens (García-Cayuela *et al.*, 2014). It is also well known that co-aggregation abilities of LAB isolates might interfere with the ability of the pathogenic microorganisms to infect the host, being able to prevent colonization by foodborne pathogens (García-Cayuela et al. 2014).

In addition, the isolate showed 11.1% ± 0.01 of adhesion capacity in the hydrophobicity test. This is relevant data for a probiotic candidate microorganism: it is a prerequisite for adhesion to the intestinal epithelium, so that it can colonize the intestine and exert a beneficial effect on the host, by exclusion and competition for sites of link with enteropathogenic bacteria (Dlamini et al. 2019).

Phenotypic characteristics such as adhesion, self-aggregation, co-aggregation and surface hydrophobicity favor microbiota colonization. However, this is a complex mechanism, which involves

many factors, but mainly aggregation and hydrophobicity allow interactions to occur between the microorganism and the host, so that it promotes beneficial health effects (García-Cayuela et al. 2014).

### Antioxidant capacity

The antioxidant capacity of LAB is important for protection against free radicals (Ren et al. 2014). The evaluation by the TBARS method showed that the isolate presented antioxidant potential and the ability to inhibit lipid peroxidation (Figure 4A). In the DPPH method, antioxidant activity was also observed: the isolate showed the ability to sequester the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, verified by a color change from violet to yellow when it comes into contact with the antioxidant substance (Figure 4B).

Similar results were found by Pieniz et al. (2014), verified that the isolates *Enterococcus* sp., *E. faecalis*, *E. faecium* and *E. hirae* presented antioxidant capacity in both methods evaluated, TBARS and DPPH. According to Wang et al. (2012), LAB that have the property of sequestering free radicals are of great interest to the food industry, as they are able to prevent deterioration and increase the shelf life of products while maintaining their nutritional value. It should be noted that the antioxidant capacity of probiotic bacteria is strain-specific, since it is related to cell wall composition, presence of enzymes and production of different metabolites (Su Oh et al. 2018).

### Safety aspects

### Production of hemolysin and activity of gelatinase, DNAse and lipase

*Lactococcus lactis* R7 showed satisfactory results in terms of safety: there was absence of DNAse, gelatinase and lipase activity, and hemolysin was not produced. Therefore, it does not present risks to human health (Domingos-Lopes et al. 2017).

### Antimicrobial susceptibility

Analysis of the results obtained for the antimicrobial susceptibility test showed that the *L. lactis* R7 isolate showed susceptibility for all the antimicrobials tested, according to CLSI standards (Table 2). A similar study by Pieniz et al. (2015) demonstrated that the *E. durans* LAB18s isolate from fresh Minas cheese presented high susceptibility for all the antimicrobials tested, thus demonstrating their safe use in food.

According to Kondrotiene et al. (2020), LAB should be investigated for their resistance to antimicrobials before being applied in commercial products, since the ability to transfer resistance genes to these compounds is a risk factor for use in food. The isolates of *L. lactis* showed great variation in resistance to antimicrobials, according to the cutoff point provided by the European Food Safety Authority (EFSA), for streptomycin and tetracycline.

### Biofilm formation

*Lactococcus lactis* R7 was evaluated for its ability to form biofilm, being classified as a non-biofilm producer. Gomes et al. (2008) evaluated the prevalence of genera of LAB isolated from food. Among them, isolates from samples of ricotta cheese were evaluated, and these presented poor capacity of biofilm formation, a result similar to that found in the present study. The formation of biofilm in the gastrointestinal tract or in the vaginal mucosa can contribute to the fixation and colonization of bacteria. However, the presence of LAB biofilm in the oral cavity contributes to the appearance of cavities. Also, in the food industry, its adhesion to surfaces and foods can lead to corrosion or deterioration (Arena et al. 2017).

## Antagonist activity of *L. lactis* R7 against selected pathogenic bacteria

The isolate *L. lactis* R7 showed antimicrobial activity against the pathogens tested through agar disk-diffusion test, as shown in Table 3. Four foodborne pathogens were tested and the isolate showed antagonistic activity against three. The largest halos were observed for *S. aureus* ATCC 25923 (12.02 mm ± 0.06) and *E. coli* ATCC 8739 (11.1 mm ± 0.15), while the lowest inhibition halo was found for *S. enteritidis* ATCC 13076 (9.5 mm ± 0.03). There was no activity against *L. monocytogenes* ATCC 19114.

There is growing research interest in new antimicrobial substances and/or microorganisms with antimicrobial potential. They are safe and natural alternatives obtained from LAB; also, they are promising biological control alternatives for the prevention or treatment of diseases of the digestive system (Jabbari *et al.*, 2017). Jang et al. (2019) evaluated the antimicrobial activity of *L. brevis* KU15153, which demonstrated inhibitory activity against *E. coli*, *S. aureus*, *L. monocytogenes* and *S. typhimurium*. Among the pathogens, *S. aureus* showed a greater zone of inhibition (18 mm ± 0.3), corroborating the results of the present study. The antimicrobial activity expressed by LAB may be due to the production of metabolites, such as acid (lactic, acetic, among others), hydrogen peroxide, diacetyl, bacteriocin, among other molecules (Oliveira et al. 2017).

## MTT cytotoxicity assay

The cytotoxic effects of the cell suspension were evaluated in VERO cell culture by performing the MTT assay, as shown in Figure 5. The results obtained in the treated cells showed no statistical difference ($p = 0.907$) in relation to the control and, even after 24 h of incubation, the metabolites resulting from the cell suspension of *L. lactis* R7 had not caused damage to the cells, which maintained the integrity of their membrane. Haghshenas et al. (2014) evaluated the cytotoxicity of *L. lactis* subsp. *lactis* 44 using the MTT test in HUVEC cells (cells of the human umbilical endothelium). They found that the metabolites, in the concentration of 30%, did not present cytotoxicity, corroborating the results of the present study.

The MTT assay is considered very sensitive for the determination of cell respiration, viability and cytotoxicity, as only viable cells are able to produce formazan products during the test, in addition to sustained bacterial fixation, even after several washes. The optical density of the remaining cells is able to remain high after absorbing the MTT stain (Vaucher et al. 2010; Poormontaseri et al. 2017; Yasmin et al. 2020).

# Conclusion

The *L. lactis* R7 isolate from ricotta cheese demonstrated probiotic potential *in vitro*, surviving during its passage through the gastrointestinal tract and presenting antioxidant capacity. In addition, it showed antimicrobial activity against foodborne pathogens, acid tolerance, homofermentative profile and absence of cytotoxicity, thus having potential for technological application in food matrices. Finally, it should be noted that the search for new isolates with promising probiotic characteristics, such as *L. lactis* R7, is extremely important for future application in food matrices and human and animal health.

# Declarations

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# Tables

Table 1. Tolerance of *L. lactis* R7 to pH, temperature, sodium chloride and phenotypic identification.

| *L. lactis* R7 | pH | | Temperature | | NaCl | | Fermentation | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH2.0 | pH9.6 | 15 °C | 45 °C | 4.0% | 6.0% | Glucose | Maltose | Ribose |
| 2 hours | + | + | + | + | + | - | | | |
| 4 hours | + | + | + | + | + | - | | | |
| 1° day | | | | | | | + | + | + |
| 4° day | | | | | | | + | + | + |
| 7° day | | | | | | | + | + | + |

Table 2. Antimicrobial susceptibility of *L. lactis* R7 and classification (CLSI, 2017).

| Antimicrobials | Classification | | |
|---|---|---|---|
| | Inhibition zones (mm) | Susceptible (mm) | [a] (S-I-R) |
| Clindamycin | 25.0 ± 0.81 | ≥ 21 | S |
| Erythromycin | 26.5 ± 0.60 | ≥ 23 | S |
| Penicillin | 28.2 ± 1.20 | ≥ 15 | S |
| Chloramphenicol | 27.0 ± 0.81 | ≥ 18 | S |
| Meropenem | 34.0 ± 1.40 | ≥ 16 | S |
| Vancomycin | 22.7 ± 0.96 | ≥ 17 | S |

[a] S, susceptible; I, intermediate; R, resistant.

Table 3. Antimicrobial activity using the culture supernatant of *L. lactis* R7 against indicator microorganisms.

| Indicator microorganisms | Agar disk-diffusion test |
|---|---|
| | —— Inhibition zone (mm) —— |
| *E. coli* ATCC 8739 | 11.1 ± 0.15 |
| *S. enteritidis* ATCC 13076 | 9.5 ± 0.03 |
| *L. monocytogenes* ATCC 19114 | nd* |
| *S. aureus* ATCC 25923 | 12.2 ± 0.06 |

* Not detected. Inhibition zones with values ≤7 mm were assumed as absence of antimicrobial activity.

# Figures

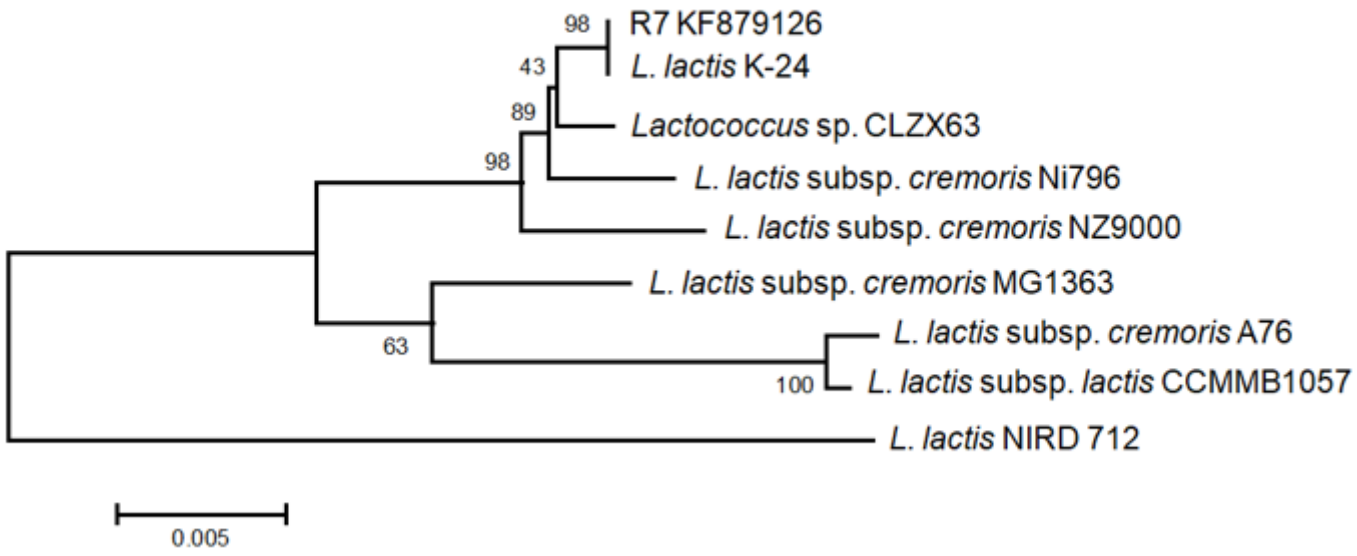
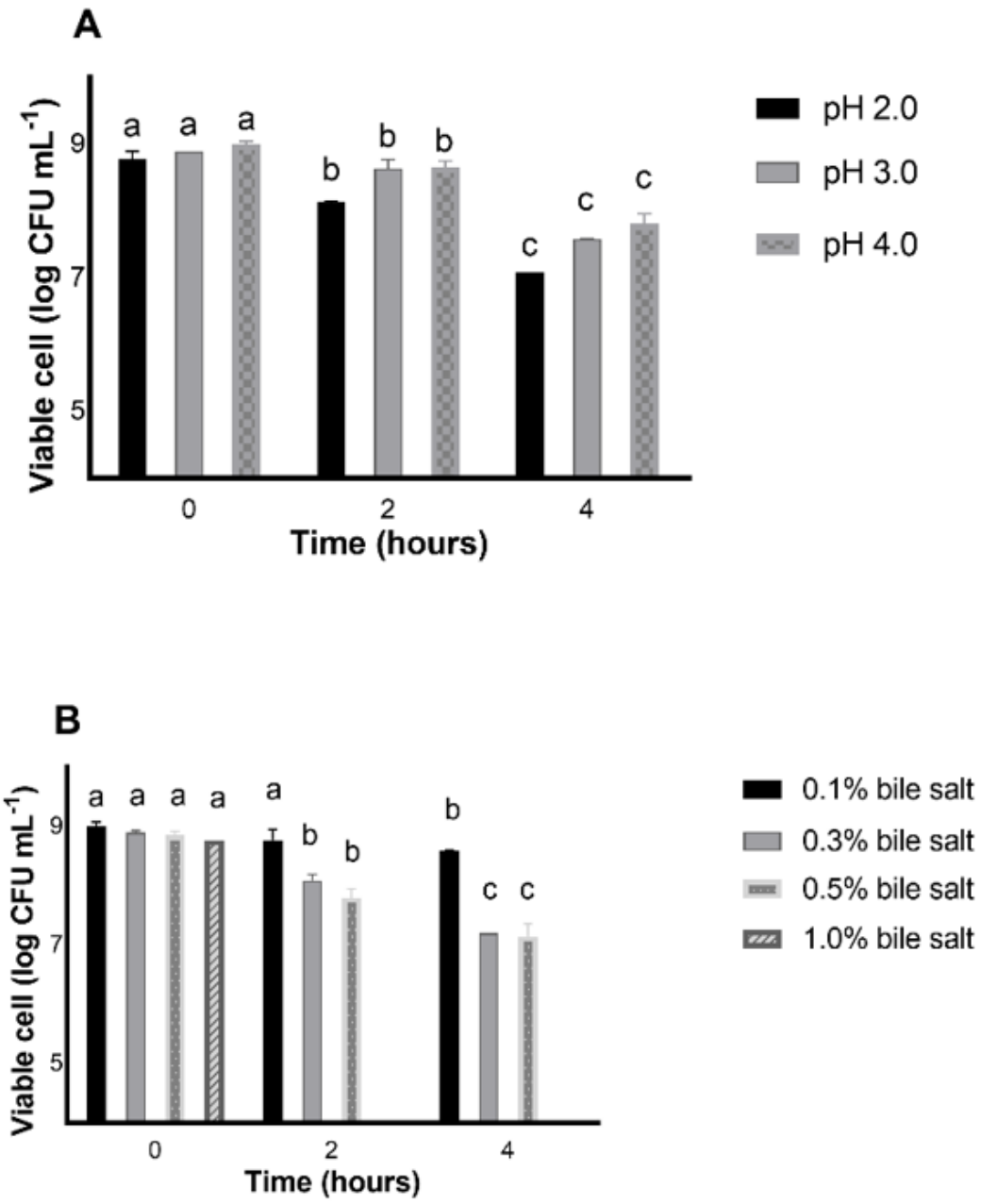


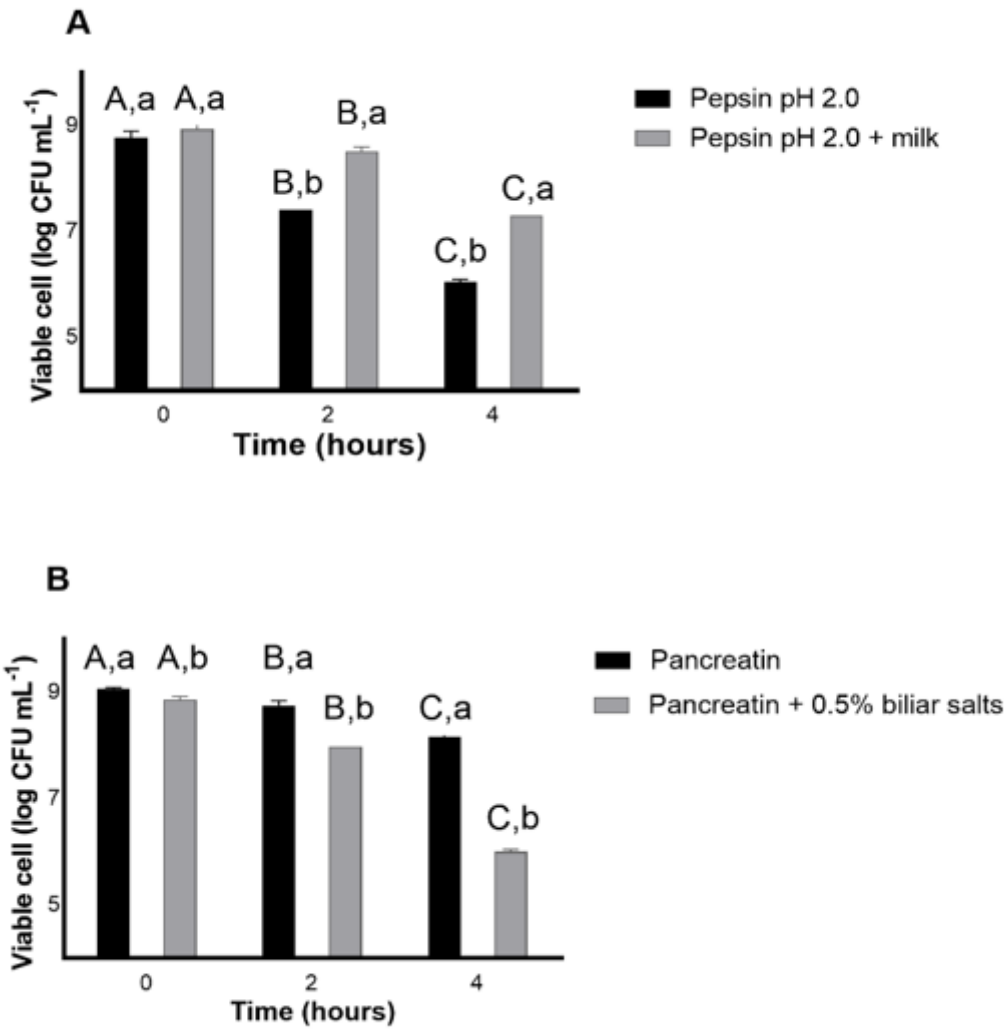

**Figure 1**

Phylogenetic tree showing evolutionary distance among the L. lactis R7 isolate (Lactococcus lactis subsp. lactis) and other Lactococcus species based on 16s rRNA gene sequence (1078bp). The bar represents the evolutionary distance value.



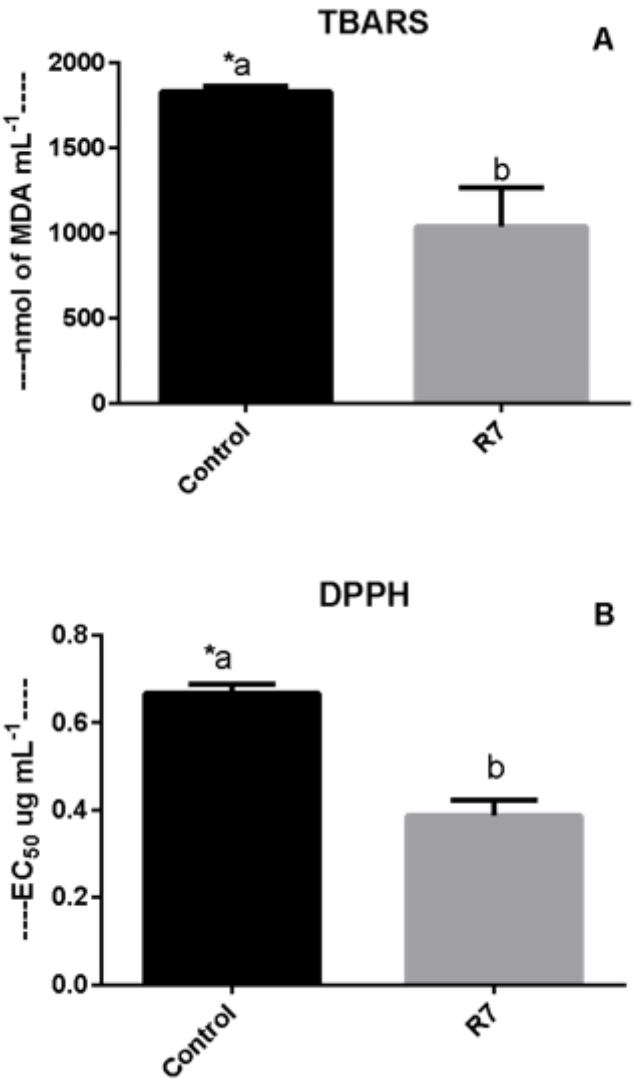

## Figure 2

Acidity tolerance in pH 2.0, 3.0 and 4.0 (A) and tolerance to bile salt at 0.1, 0.3, 0.5 and 1.0% (B) of Lactococcus lactis R7. a–c Small superscript letters represent statistical difference between times, compared for the same pH or bile salt concentration.



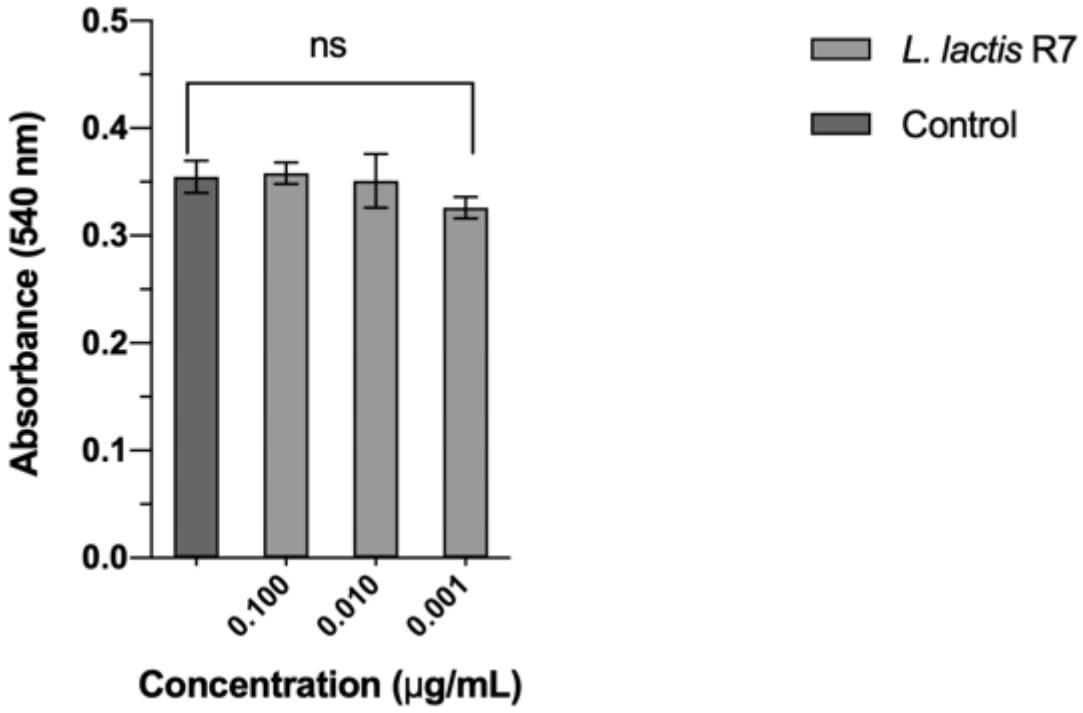

## Figure 3

Tolerance to gastric juice simulated with pepsin pH 2.0 or pepsin pH 2.0 + milk (A) and exposure to intestinal fluid in the presence or absence of 0.5% bile salts (B) of Lactococcus lactis R7. a–b Superscript lowercase letters represent statistical difference in the same test when comparing the hours of exposure. A–C Superscript capital letters represent statistical difference in the different tests within the same hour of exposure.




## Figure 4

Inhibition of lipid peroxidation by TBARS method (A) and analysis of antioxidant capacity of the crude supernatant of Lactococcus lactis R7 by DPPH method (B). Results were expressed as mean ± standard deviation. * Values with the same letters do not present significant statistical differences between groups.




## Figure 5

Cytoxicity assay (MTT) results for VERO cells exposed to 0.1, 0.01 and 0.001 µg.mL-1 of the bacterial suspension of L. lactis R7. ns Values do not present significant statistical differences between the treatments.