CADERNO DE CIÊNCIAS AGRÁRIAS
Agrarian Sciences Journal

ICA INSTITUTO DE CIÊNCIAS AGRÁRIAS

UFMG

In vitro evaluation of the probiotic potential of microorganisms isolated from functional commercial fermented milks

João Victor Ferreira Campos[1], Mariana Oliveira Silva[2], Ana Carolina Alves Vieira[3], Luiza Mota de Faria[4], Gustavo Lucas Costa Valente[5], Priscila Mara Rodarte Lima e Pieroni[6], Marcelo Resende de Souza[7], Jacques Robert Nicoli[8], Leonardo Borges Acurcio[9]*

DOI: https://doi.org/10.35699/2447-6218.2021.35380

Abstract

Fermented milk is one of the carriers of probiotics, which is a Greek derived term meaning "pro-life". Probiotics are live microorganisms that, when administered in adequate and daily amounts, confer benefits to host's health. In this work, four *in vitro* tests were performed with six lactobacilli samples from commercial fermented milks: sensitivity test to gastric pH, sensitivity test to intestinal bile salts, antagonism test against pathogens and susceptibility test to antimicrobials. The tested samples were resistant to gastric pH (2.0) and some suffered growth inhibition in the sensitivity tests to bile salts (0.3%), in two different methods, reducing growth in about 45% or 1 Log_{10} reduction. All pathogenic bacteria tested *(Escherichia coli, Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus*) were antagonized by the supernatant (in MRS) of *Lactobacillus* spp. isolated from commercial fermented milks as well as in spot on the lawn test. Regarding antimicrobial susceptibility, samples presented a diverse profile, with prevalence of sensitivity to antimicrobials of clinical relevance, although there was high resistance profile regarding cephalosporins. In view of the *in vitro* tests performed, samples showed a satisfactory probiotic potential, as expected. However, some samples showed a slightly superior performance, such as *Lactobacillus casei* Shirota from Yakult, *L. casei* Defensis from Actimel and *L. paracasei* ST11 from Chamyto.

Keywords: Antagonism. *Lactobacillus*. Resistance to gastrointestinal transit.

Avaliação *in vitro* do potencial probiótico de micro-organismos isolados de leites fermentados funcionais comerciais

[1]Centro Universitário de Formiga (UNIFOR-MG) – Formiga, MG.
https://orcid.org/0000-0003-0902-0000

[2]Universidade Federal de Minas Gerais (UFMG) – Belo Horizonte, MG.
https://orcid.org/0000-0002-0537-1666

[3]Universidade Federal de Minas Gerais (UFMG) – Belo Horizonte, MG.
https://orcid.org/0000-0002-9284-8054

[4]Centro Universitário de Formiga (UNIFOR-MG) – Formiga, MG.
http://orcid.org/0000-0001-9726-056X

[5]Universidade Federal de Minas Gerais (UFMG) – Belo Horizonte, MG.
http://orcid.org/0000-0001-8256-0383

[6]Centro Universitário de Formiga (UNIFOR-MG) – Formiga, MG.
http://orcid.org/0000-0001-6621-6777

[7]Universidade Federal de Minas Gerais (UFMG) – Belo Horizonte, MG.
http://orcid.org/0000-0001-7071-7304

[8]Universidade Federal de Minas Gerais (UFMG) – Belo Horizonte, MG.
https://orcid.org/0000-0003-2390-2608

[9]Centro Universitário de Formiga (UNIFOR-MG) – Formiga, MG.
https://orcid.org/0000-0002-2981-5479

Corresponding author. leoacurcio@uniformg.edu.br.

Recebido para publicação em 27 de julho de 2021. Aceito para publicação em 28 de setembro de 2021
e-ISSN: 2447-6218 / ISSN: 2447-6218. **Atribuição CC BY.**

Resumo

O leite fermentado é um dos veiculadores dos probióticos, que é um termo derivado do grego que significa "a favor da vida". Probióticos são microrganismos vivos, que quando administrados em quantidades adequadas e diárias, conferem benefícios à saúde do hospedeiro. Neste trabalho foram realizados quatro testes *in vitro* com amostras de lactobacilos de seis leites fermentados comerciais: teste de sensibilidade ao pH gástrico, teste de sensibilidade aos sais biliares intestinais, teste de antagonismo contra agentes patogênicos e teste de suscetibilidade aos antimicrobianos. As amostras testadas foram resistentes ao pH gástrico (2.0) e algumas sofreram inibição do crescimento nos testes de sensibilidade aos sais biliares (0,3%), em dois tipos de teste *in vitro* diferentes, com redução de aproximadamente 45% no crescimento ou cerca da 1 Log_{10}. Todas as bactérias patogênicas testadas (*Escherichia coli, Salmonella enterica* sorovar Typhimurium e *Staphylococcus aureus*) foram antagonizadas pelo sobrenadante (em MRS) dos *Lactobacillus* spp. isolados de leites fermentados comerciais assim como em teste de antagonismo to tipo *spot on the lawn*. Quanto à suscetibilidade aos antimicrobianos, as amostras apresentaram perfil diversificado com predominância para a sensibilidade a antimicrobianos de importância clínica, apesar do elevado perfil de resistência às cefalosporinas observado. Frente aos testes *in vitro* realizados, as amostras apresentaram potencial probiótico satisfatório.

Palavras-chaves: Antagonismo. *Lactobacillus*. Resistência ao trato gastrointestinal.

Introduction

Concerned about a healthier life, more and more people have been looking for better life habits, associating physical exercise with a balanced diet (Berti *el al.*, 2017). Knowing this, food industry, aware of consumer preferences, has raised its concern over healthy and functional food and beverage market (Granato *et al.*, 2010). In this context, fermented milk or fermented dairy product stand out as an attractive vehicle for probiotic cultures (Barat; Ozcan, 2017).

The International Dairy Federation defines fermented dairy product as a dairy product prepared from skimmed or non-skimmed milk with specific cultures (Panesar, 2011). The main bacteria used in its production are usually lactobacilli, being responsible for the sensory characteristics of the product and the reduction of its pH. Addition of probiotic microorganisms in the product reinforces its functional properties by increasing its nutritional and therapeutic value, also resulting in the improvement of its sensory characteristics (Sharifi-Rad *et al.*, 2020).

Probiotics have its' beneficial properties only when administered in adequate amounts and in viable conditions (FAO/WHO, 2002). So, a minimum viable quantity for probiotics was established, ranging from 10^8 and 10^9 Colony Forming Units (CFU) in the daily recommendation of the product (ready for consumption). In addition, for a product to be classified as a probiotic, used culture must have resistance to bile salts and gastric acidity confirmed by laboratory tests, according to Brazilian legislation (Brasil, 2018). Resistance to gastric and intestinal injuries is of great importante in probiotic selection, since a minimum of 10^6 CFU is required for a probiotic sample to exert is possible beneficial effects. If not resistant, bacterial count may reduce drastically and probiotic effect will not occur (Vinderola; Reinhemer, 2003).

The objective of this work was to evaluate the *in vitro* fuctional features of microorganisms isolated from commercial functional fermented milks. For this, *in vitro* sensitivity of microorganisms to gastric juice and bile salts was evaluated, as well as antagonistic activity of microorganisms against pathogenic bacteria and susceptibility to antimicrobials.

Material and Methods

Commercial functional fermented milks found in retail chain of Minas Gerais were evaluated, namely: Actimel from Danone (Danone, Paris, France), Batavinho from Batavo (Batavo, Carambeí, Brazil), Chamyto from Nestlé (Nestlé, Vevey, Switzerland), Itambé from Itambé (Itambé Alimentos, Belo Horizonte, Brazil), Vigor from Vigor (Vigor Alimentos, São Paulo, Brazil), and Yakult from Yakult (Yakult, Tokyo, Japan), from which single species of probiotic *Lactobacillus* sp. were isolated after count, which is described below. All products were bought within expiry date, under refrigeration (<7°C) and were maintained at this temperature until experimentation.

Count of viable microorganisms was carried out from six different samples of commercial fermented milk. Initially, a serial dilution of up to 10^{-5} of 0.1mL of each sample was made in 0.9mL of sterile peptone saline (0.9% NaCl, 0.1% peptone). Afterwards, 0.1mL of the solution obtained from each dilution was plated with the aid of the Drigalski's loop. Solution was then distributed over the surface of petri dishes containing MRS (de Man Rogosa and Sharpe - MRS) agar (Difco, Detroit, USA). Plates were incubated aerobically for 24 hours at 37°C. Colonies with different morphotypes were subjected to Gram staining and catalase tests, in order to confirm it as lactobacilli.

Antibiogram was performed according to the technique of antimicrobial susceptibility by the principle

of drug diffusion in disc. Discs with pre-defined drug concentrations were used and the diameters of the halos formed by inhibition were measured following the one proposed by Charteris *et al.* (1998a).

Each microorganism was transferred to a test tube containing 3.5mL of 0.9% saline until the concentration equivalent to the 0.5 McFarland scale (corresponding to an estimated population of 10^8 CFU/mL). Culture was then spread on a pizza-type Petri dish (14cm diameter) with a sterile swab until the entire surface containing MRS agar (Difco) was covered. Then, in an equidistant manner, 10 disks (Laborclin, Pinhais, Brasil) containing antimicrobials from different chemical groups were distributed. Drugs belonging to the following chemical groups were used:

Cell wall synthesis inhibitors, with bactericidal action, such as beta-lactams: penicillin (PEN, 10U), ampicillin (AMP, 10μg). Glycopeptides: such as vancomycin (VA, 30μg). Third generation cephalosporins: ceftriaxone (CRO, 30μg), cefoxitin (CFO, 30μg). Protein synthesis inhibitors, with bacteriostatic action, such as tetracyclines: tetracycline (TE, 30μg); as well as aminoglycosides: gentamicin (GEN, 10μg), chloramphenicol (CLO, 30μg) and streptomycin (EST, 30μg). Cell multiplication inhibitors, with bactericidal action, such as the third generation quinolones: ciprofloxacin (CIP, 5μg).

After the distribution of disks, Petri dishes were incubated in aerobiosis at 37°C for 48 hours. For quality control of disks containing antimicrobials, a sample of *Escherichia coli* ATCC 25922 was used. Finally, diameters of the inhibition halos were read using a Mitutoyo digimatic caliper (Mitutoyo, Suzano, Brasil). The test was performed in duplicate with two repetitions. Characterization of the antimicrobial susceptibility profiles of the evaluated samples was carried out according to Charteris *et al.* (1998a).

Before the next test was carried out, lactic acid bacteria isolated from each fermented milk was grown in 5mL of MRS broth (Difco) and incubated for 48 hours at 37°C under aerobic conditions. Then, *in vitro* antagonism test against pathogenic microorganisms was performed in two different ways, each technique being used for every six samples.

First method: five microliters of each microorganism culture were placed on the center of the surface of a Petri dish, containing MRS agar (Difco), which was incubated under aerobiosis at 37°C for 48 hours. After 48 hours, the plates were removed from the incubation chambers with the spots in the center of the plate grown. Chloroform was placed on the plate covers and left for 30 minutes under ultraviolet (UV) light to perform its action. With this process, the microorganisms that grew in the spots were eliminated, allowing the evaluation of supposed inhibitory substances produced by the bacteria and released in the culture medium. Next step consisted of placing 3.5ml of soft agar (0.75% BactoAgar, Difco, in broth of Brain Heart Infusion - BHI, or MRS, Difco) with revealing bacteria. The methodology used in this test was adapted from Acurcio *et al.* (2014).

Revealing bacteria were the following pathogenic microorganisms: *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATTCC 14028, *Shigella flexneri* ATCC 12022 and *Staphylococcus aureus* ATCC 29213. 10μL of the microorganisms that had been previously incubated were transferred to semi-solid agar and poured onto MRS agar plates containing the previously described spots.

Finally, plates were incubated at 37°C for 48 hours, under aerobiosis. Inhibition halos were read using a digimatic caliper (Mitutoyo). This test was performed in duplicate - the same revealing microorganism applied to two plates with the same isolated microorganism tested (spot) - with two replicates.

Second method: *in vitro* antagonism methodology by Acurcio *et al.* (2017) was used, with adaptations. Initially, microorganisms isolated from the six samples of fermented milk were grown in MRS broth (Difco) at a temperature of 37°C for 24 hours, under aerobic conditions. Then, 1mL of each probiotic sample was pipetted into three different microtubes. Microtubes were centrifuged at 5.000g for 5 minutes and, supernatants were obtained. The pH of each supernatant was measured and its microfiltration was carried out, in order to ensure the exclusive action of culture supernatants.

The reference microorganisms (*Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Staphylococcus aureus* ATCC 29213) were inoculated in BHI broth (Brain Heart Infusion) and subsequently incubated at 37°C for 24 hours. Afterwards, in three microtubes of each pathogenic sample, 4% (v/v) solutions were prepared containing 960μl of BHI broth (Difco) added, individually with 40μl of one of the pathogenic microorganisms. Then, 100μL of the probiotic supernatant was transferred to an ELISA plate with 96 wells and another 100μL of the solutions described above. The plate was then incubated in a spectrophotometer (Microplate Spectrophotometer System SpectraMax 340 - Molecular Devices, San Jose, USA) for 12 hours at 37°C. The OD 620nm reading at every 30 minutes determined the absorbance of the culture.

To calculate the percentage of growth inhibition, the GraphPad Prism 6.01 (GraphPad, San Diego, USA) program was used. The formula (1-AT / CT) x 100, with AT corresponding to the area under the growth curve of the revealing pathogenic microorganism in probiotic supernatant and CT correspond to the area under the growth curve of the control pathogenic microorganism, was adapted form Andrade *et al.* (2014). The control consisted of adding the solutions of pathogenic samples in pure MRS broth (Difco), in which there was no previous

growth of any probiotic microorganism. The tests were performed in triplicate, with two repetitions.

Evaluation of the sensitivity or resistance of microorganisms to gastric pH and intestinal bile salts were also carried out in two different ways.

First technique (gastric simulation): To measure the sensitivity to gastric pH, the technique described by Santos *et al.* (2016) was primarily used. The isolated microorganisms were activated in MRS broth (Difco) and subsequently incubated for 48 hours at 37°C under aerobic conditions. In order to define the control growth of each of the lactobacilli samples, the microorganisms were plated prior to the test in the serial dilutions at 10^{-4} and 10^{-6} on MRS agar (Difco) (incubation at 37°C, for 48 hours, aerobically). Later, 1ml of each sample was centrifuged in 2ml microtubes at 5.000g for 5 minutes. The obtained supernatant was discarded and the samples were exposed to 1 ml of 0.9% saline solution (pH 2.0, 0.3 w/v of pepsin), for one hour. After the determined time, a plating based on serial dilutions at 10^{-4} and 10^{-6} on MRS agar (Difco) was performed, with the purpose of evaluating the viability of the cells. Finally, the plates were incubated at 37°C for 48 hours, aerobically. The results obtained were compared with the results of the plating done before the test to observe the behavior of the microorganisms studied in an artificial stomach environment.

Second technique (gastric simulation): Adaptations were performed over methodolgy proposed by Acurcio *et al.* (2014). First, the isolated microorganisms were activated in MRS broth (Difco) and incubated for 24 hours at 37°C, under aerobic conditions. After incubation, 1mL of each sample of the activated microorganisms was added in two different microtubes that were centrifuged at 5.000g for 5 minutes. The supernatants formed after centrifugation were discarded and the pellet obtained was suspended in saline with pH 2.0 and 0.3 w/v of pepsin (gastric pH) or 7.0 (control). After that, the samples were incubated at 37°C for one hour and centrifuged again under the same conditions mentioned above. After centrifugation, the pellet was suspended in MRS (Difco). 2% (v/v) inoculum was also prepared in MRS broth (Difco). The sample was then distributed in a 96-well ELISA plate, with 200μl of each sample after passage through pH 2.0 (gastric pH) and pH 7.0 (control).

The plate was incubated in a spectrophotometer (Microplate Spectrophotometer System SpectraMax 340 - Molecular Devices) at 37°C for 12 hours and the absorbance of the culture determined by reading in OD 620nm every 30 minutes. The percentage of growth inhibition was calculated using the GraphPad 6.01 program (GraphPad Software) as a tool using the formula (1-SG / CT) x 100, with SG corresponding to the area under the growth curve of bacteria treated with artificial gastric juice and CT, the control. The tests were performed in triplicate, with two repetitions.

First technique (intestinal bile salts): To measure sensitivity to bile salts, the technique described by Santos *et al.* (2016) was adapted. The isolated microorganisms were activated in MRS broth (Difco) and incubated at 37°C under aerobiosis for 48 hours. Previously to the assay, the microorganisms were plated at serial decimal dilutions at 10^{-4} and 10^{-6} on MRS agar (Difco) in order to define the control growth of each of the lactobacilli samples (incubation at 37°C, for 48 hours, aerobically). Subsequently, 1ml of each sample was centrifuged in 2mL microtubes at 5.000g for 5 minutes. The supernatant formed was discarded and the samples exposed to 1ml of MRS broth solution (Difco) containing 0.3% (w/v) of bile salts (Oxgall, Difco) for six hours. At the end of the six hours, plating was done from decimal dilutions serialized at 10^{-4} and 10^{-6} on MRS agar (Difco) in order to assess the viability of the cells. Finally, plates were incubated at 37°C for 48 hours in aerobiosis. The results found were compared with the plating results made prior to the test to observe the behavior of the microorganisms studied in an artificial intestinal environment.

Second technique (intestinal bile salts): To assess the resistance of microorganisms isolated from fermented milk to bile salts, the tests were based on the methodology described by Acurcio *et al.* (2017). First, the isolated microorganisms were activated in MRS broth (Difco) and incubated in an aerobic condition at 37°C for 24 hours. After incubation, the activated microorganisms were placed in microtubes at a 4% (v/v) dilution in MRS broth (Difco). Then, 100μL were transferred to one well on the 96-well ELISA plate containing 100μl of pure MRS broth (Difco) and another 100μL were transferred to another well on the same plate containing MRS broth (Difco) with 0.6% (w/v) of bile salts (Oxgall). To determine the absorbance of the culture, the reading of OD 620nm was performed, every 30 minutes for 12 hours.

In the end, the inoculum of samples was 2% and the concentration of bile salts 0.3% in the wells, simulating, *in vitro*, intestinal environment. To calculate the percentage of growth inhibition, the program GraphPad Prism 6.01 (GraphPad Software) was used, which carried out the determination by the formula (1-SB / CT) x 100, with SB corresponding to the area under the growth curve of the control bacteria treated with bile salts and CT, the control. The tests were performed in triplicate, with two repetitions.

Results and discussion

Regarding microorganisms' count (Table 1) it was possible to observe if counts were between 10^6 to 10^9 CFU/ml. According to Vinderola and Reinhemer (2003), recomendend dose would be approximately 10^8 CFU per dose. Smaller values are accepted, once their effectiveness is proven (Minelli and Benini, 2008). Considering the approximate consumption of 100g of product, the product with the lowest count would be able to achieve at least the necessary count for its functional claim.

Table 1 – Average count in Log_{10} (CFU/mL on MRS agar) of lactobacilli isolated from functional fermented milks

Sample	MRS count in Log_{10} (CFU/mL) ± SD *
YA – *L. casei* Shirota (Yakult)	8,78±0,02
AC – *L. casei* Defensis (Actimel)	8,60±0,01
CH – *L. paracasei* ST11 (Chamyto)	8,40±0,07
IT – *L. casei* (Itambé)	7,28±0,02
VI – *L. casei* (Vigor)	6,34±0,02
BA – *L. acidophilus* (Batavo)	5,96±0,04

* SD = Standard Deviation.

According to Forssten and Ouwehand (2020), cell viability is the fundamental test for assessing the effectiveness of lactic acid bacteria as probiotics. Probiotic bacteria must be able to withstand stress from gastrointestinal tract, such as gastric pH and bile salts in the small intestine. These bacteria need to resist such injuries to be able to exercise their therapeutic benefits.

Regarding *in vitro* resistance test to artificial gastric juice, it was possible to observe that all samples (Table 2) were able to survive artificial gastric juice (pH 2.0). Similar results were found by Hoque *et al.* (2010), where all isolated and evaluated microorganisms were able to survive at pH 2.2. Costa *et al.* (2013), in a similar study, found a low percentage of inhibition to artificial gastric juice and a high percentage of inhibition to bile salts, pointing the satisfactory results of this study. Maragkoudakis *et al.* (2006) also observed that, within two hours, with pepsin, artificial gastric juice did not have a great effect on the count of probiotic microorganisms in commercial functional fermented milks.

Table 2 – Percentage (%) of *in vitro* inhibition of gastric juice (pH 2.0) and bile salts (0.3% Oxgall) of lactobacilli isolated from functional fermented milks

Samples	In vitro inhibition (functional test)	
	Gastric juice (%)	Bile salts (%)
YA	No inhibition	35,77
AC	No inhibition	39,12
CH	No inhibition	41,36
IT	No inhibition	45,31
VI	No inhibition	45,71
BA	No inhibition	43,77

Table 2 showed, when tested by turbidimetry in spectrophotometry, something around inhibition of about 40% of the growth of lactobacilli with probiotic claim isolated from fermented milks. In the plating before and after exposure to bile salts at 0.3%, we can see an even more encouraging result, with a low reduction in counts (average of 13% reduction – Table 3). With regard to artificial gastric juice (pH 2.0), there was also good resistance to this TGI challenge (average of 8% reduction – Table 3). Silva *et al.* (2013) found higher percentages of inhibition from bile salts of lactobacilli candidates. Costa *et al.* (2013), in turn, observed in samples of lactic acid bacteria evaluated as to their potential, mostly, sensitivity to bile salts. This reinforces the individuality of the probiotic potential of the sample, despite the similarity of the tested species. Caillard and Lapointe (2017) found samples of *Lactobacillus* spp. with probiotic potential tolerant and not tolerant to gastric acid (pH 2.0)

The ability of certain samples of lactic acid bacteria, such as *Lactobacillus casei*, to survive in acidic environments is extremely important for the functionality of these microorganisms during bioprocessing. Acid adaptation experiments with *L. casei* ATCC 334 demonstrated that the induction of acid tolerance response can be triggered by transient exposure to various sub-lethal pH values. When faced with acid stress, bacteria can act to counter the influx of protons, increasing the rigidity and compactness of the cytoplasmic membrane (Broadbent *et al.*, 2010).

Table 3 – Percent inhibition (CFU/mL and %) of six samples of lactic acid bacteria isolated from commercial fermented milks, in gastric pH (2.0) and bile salts (0.3% Oxgall)

Samples	Count (CFU/mL) - Percentage reduction (%)		
	Initial	Artificial gastric juice	Bile salt
YA	$3,95x10^9$	$9,10x10^8$ - 8,72	$2,82x10^8$ - 14,48
AC	$6,00x10^9$	$8,87x10^8$ - 6,64	$3,25x10^8$ - 11,94
CH	$6,55x10^9$	$9,13x10^8$ - 8,04	$2,48x10^8$ - 13,21
IT	$5,55x10^9$	$9,13x10^8$ - 8,49	$2,86x10^8$ - 12,96
VI	$5,30x10^9$	$9,70x10^8$ - 7,58	$2,30x10^8$ - 14,01
BA	$5,05x10^9$	$7,80x10^8$ - 8,36	$2,24x10^8$ - 13,94

Another factor that has an important effect on the gastric tolerance of some samples is the presence of milk proteins, alone and/or in combination. Studies have shown that *L. casei* 212.3 was able to survive gastric juice in the presence of sodium caseinate, protein whey and a combination of these (Charteris *et al.*, 1998b).

The *in vitro* antimicrobial susceptibility test performed on some lactic acid bacteria (Table 4) showed that all microorganisms were resistant to three antimicrobials (ceftriaxone, gentamicin and vancomycin) and sensitive to five others (ampicillin, ciprofloxacin, chloramphenicol, penicillin and tetracycline). It is desirable that a probiotic candidate is sensible to all antimicrobials, in order to avoid genetic transference do pathogenic microorganisms. However, presence of resistance, if acquired (not intrinsic) brings concern, as it may be involved in transference of resistance of antimicrobials of clinical use, such as cephalosporins, to gut pathobiont bacteria (Sharma *et al.*, 2014). For the other antimicrobials tested (streptomycin and cefoxitin), there was a diverse susceptibility profile. Divergent results were observed by Cebeci and Gurakam (2003), in which only 27% of the evaluated *Lactobacillus plantarum* samples were resistant to gentamicin. Costa *et al.* (2013) revealed that vancomycin resistance occurred in all samples of *Lactobacillus* spp. tested, which was also observed in other studies (Anisimova; Yarrulina, 2019; Guo *et al.*, 2017), corroborating the statement that lactobacilli can have intrinsic resistance to vancomycin (Teuber *et al.*, 1999).

Table 4 – Antimicrobial susceptibility profile of six samples of lactic acid bacteria isolated from commercial fermented milks

Samples	Antimicrobial									
	TET	AMP	EST	CRO	CLO	CIP	CFO	GEN	VAN	PEN
YA	S	S	S	M	S	S	R	R	R	S
AC	S	S	M	S	S	S	R	R	R	S
CH	S	S	S	S	S	S	R	S	R	S
IT	S	S	R	S	S	S	R	R	R	S
VI	S	S	M	M	S	S	R	R	R	S
BA	S	S	M	M	S	S	R	R	R	S

TET = tetracycline, AMP = ampicillin, EST = streptomycin, CRO = ceftriaxone, CLO = chloramphenicol, CIP = ciprofloxacin, CFO = cefoxitin, GEN = gentamincin, VAN = vancomycin, PEN = penicillin. S = sensitive, M = moderately sensitive, R = resistant.

The *in vitro* antagonism test (from supernatant) (Table 5) showed antagonistic activity of all samples evaluated against the three pathogenic bacteria tested (*Escherichia coli*, *Salmonella* Typhimurium and *Staphylococcus aureus*). In sample BA there was a higher percentage of antagonism (77.21%) against *E. coli*, while sample YA showed a high percentage of inhibition (86.65%) against *Salmonella* Typhimurium. In turn, samples CH and VI showed 72.85 and 72.66% inhibition against *S. aureus*, respectively. Results by Silva *et al.* (2013) also showed strong antagonistic activity against seven pathogenic bacteria tested (which included pathogenic samples of the species selected here), as well as those of Maragkoudakis *et al.* (2006), who observed the inhibition of certain probiotic samples of *Lactobacillus* spp., as well as pathogenic *E. coli* and *S.* Typhimurium.

Table 5 – Percentage (%) of antagonism (inhibition) by the supernatant (in MRS) of lactobacilli isolated from functional fermented milks against relevant pathogenic microorganisms

Sample	pH + SD*	Inhibition of growth*		
		E. coli	*S.* Typhimurium	*S. aureus*
YA	3,96±0,10	76,88	86,65	71,92
AC	4,08±0,06	70,72	82,74	70,16
CH	4,00±0,09	73,35	83,72	72,85
IT	4,04±0,07	69,50	82,27	56,85
VI	3,96±0,08	72,08	84,15	72,66
BA	4,03±0,11	77,21	81,72	70,82

*Inhibition values are represented as a percentage (%). SD = Standard Deviation.

Some strains of lactobacilli are capable of producing potent antimicrobial compounds, such as bacteriocins, antimicrobial peptides and organic acids, such as lactic acid (Mohanty; Saini; Mohapatra, 2017), which can inhibit bacteria with a deteriorating and/or pathogenic character, including those from the following genera: *Salmonella*, *Escherichia*, *Pseudomonas* and *Staphylococcus* (Castro *et al.*, 2011).

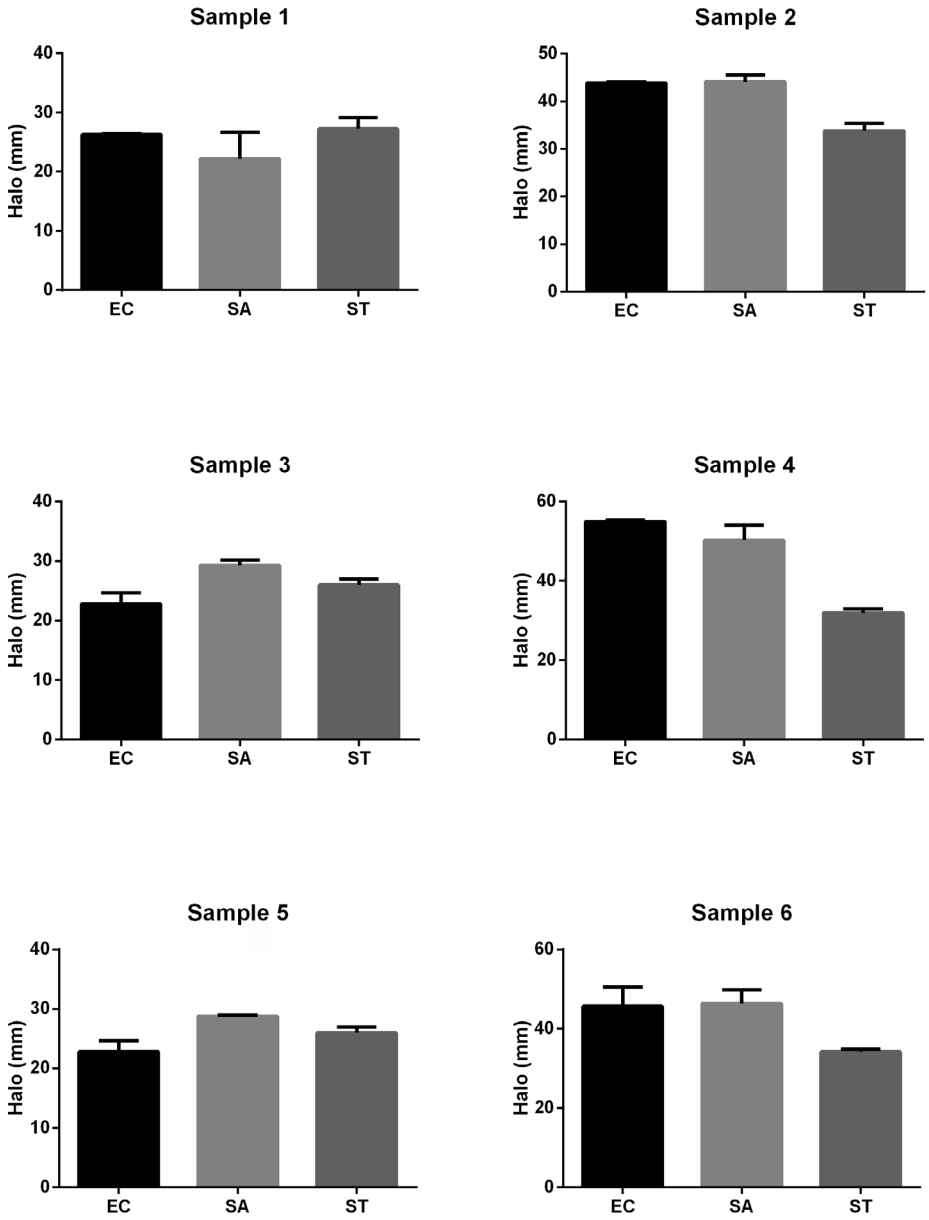
The *in vitro* antagonistic effect, by the spot on the lawn technique, of samples of lactic acid bacteria against pathogenic microorganisms (*Escherichia coli, Staphylococcus aureus* and *Salmonella* Typhimurium) was also measured, showing that all tested samples were able to produce halos of inhibition against pathogens (Graph 1).

Average of inhibition halos for each pathogenic bacteria were more expressive over *Staphylococcus aureus* and *Escherichia coli. Salmonella* Typhimurium, in turn, showed a quantitative lower inhibition by all probiotic tested samples, which is not necessarily a sign for lower effectiveness. Slightly different results were found by Costa *et al.* (2013), in which the lowest quantitative inhibition against pathogenic microorganisms occurred against *S. aureus*. Guedes Neto *et al.* (2005), in turn, found a diversified response on the part of the lactobacilli prospecting regarding its probiotic characteristics. These data reinforce that, in the context of probiotic potential against the inhibition of pathogens, lactobacilli tested in vitro in the present study were able to inhibit pathogenic microorganisms of relevance to food and diseases associated with food, especially those of animal origin.

Conclusion

Microorganisms carried by commercial fermented milks confirmed, as expected, a favorable probiotic potential. Tested samples presented outstanding resistance do *in vitro* gastric environment and great resistance to biliary salts *in vitro* challenge (average of 40% of inhibition). Intestinal injury challenge did not reduce more than one Log_{10} of probiotic population, which is desirable. Samples performed remarkable *in vitro* antagonism against pathogenic tested strains. Resistance to cephalosporins is a concern since they are probably not intrinsic, with the risk of horizontal transmission to pathobiont microorganisms. So, probiotic features of commercial strains performed accordingly, with a discrete quantitative superiority of samples from Actimel, Chamyto and Yakult.

Graph 1 – Average results of *in vitro* antagonism tests (mm of inhibition halo) of probiotic lactic acid bacteria against pathogenic microorganisms



EC = *Escherichia coli;* SA = *Staphylococcus aureus;* ST = *Salmonella* Typhimurium. Bars represent standard deviation. Sample 1: BA, Sample 2: YA, Sample 3: VI, Sample 4: CH, Sample 5: IT, Sample 6: AC.

References

Acurcio, L. B.; Souza, M. R.; Nunes, A. C.; Oliveira, D. L. S.; Sandes, S. H. C.; Alvim, L. B. 2014. Isolation, enumeration, molecular identification and probiotic potential evaluation of lactic acid bacteria isolated from sheep milk. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 66:940–948. DOI: https://doi.org/10.1590/1678-41625796.

Acurcio, L. B.; Bastos, R. W.; Sandes, S. H. C.; Guimarães, A. C. C.; Alves, C. G.; Reis, D. C.; Wuyts, S.; Nunes, A. C.; Cassali, G. D.; Lebeer, S.; Souza, M. R.; Nicoli, J. R. 2017. Protective effects of milk fermented by *Lactobacillus plantarum* B7 from Brazilian artisanal cheese on a *Salmonella enterica* serovar Typhimurium infection in BALB/c mice. Journal of Functional Foods. 33:436–445. DOI: https://doi.org/10.1016/j.jff.2017.04.010.

Andrade, C. R. G.; Souza, M. R.; Penna, C. F. A. M.; Acurcio, L. B. Sant'Anna, F. M.; Castro, R. D.; Oliveira, D. L. S. 2014. Propriedades probióticas in vitro de *Lactobacillus* spp. isolados de queijos minas artesanais da Serra da Canastra - MG. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 66:1592–1600.DOI: https://doi.org/10.1590/1678-6781.

Anisimova, E. A., Yarullina, D. R. 2019. Antibiotic Resistance of *Lactobacillus* Strains. Current Microbiology, 76:1407–1416. DOI: https://doi.org/10.1007/s00284-019-01769-7.

Brasil. 2018. Resolução da Diretoria Colegiada nº 241. Dispõe sobre os requisitos para comprovação da segurança e dos benefícios à saúde dos probióticos em alimentos. Ministério da Saúde, Agência de Vigilância Sanitaria, Brasília, 2018.

Broadbent, J. R.; Larsen, L. R.; Deibel, V.; Stelle, J. L. 2010. Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress. Journal of Bacteriology, 192:2445–2458. DOI: https://doi.org/10.1128/JB.01618-09.

Barat, A.; Ozcan, T. 2017. Growth of probiotic bacteria and characteristics of fermented milk containing fruit matrices. International Journal of Dairy Technology, 71:120–129. DOI: https://doi.org/10.1111/1471-0307.12391.

Berti, C.; Agostini, C.; Davanzo, R.; Hyppönen, E.; Isolauri, E.; Meltzer, H. M.; Steeger-Theunissen, R.P.M., Cetin, I. 2017. Early-life nutritional exposures and lifelong health: immediate and long-lasting impacts of probiotics, vitamin D, and breastfeeding, Nutrition Reviews, 75:83–97. DOI: https://doi.org/10.1093/nutrit/nuw056.

Caillard, R.; Lapointe, N. 2017. In vitro gastric survival of commercially available probiotic strains and oral dosage forms, International Journal of Pharmaceutics, 519:125–127. DOI: https://doi.org/10.1016/j.ijpharm.2017.01.019.

Castro, M. P.; Palavecino, N. Z.; Herman, C.; Garro, O. A.; Campos, C. A. 2011. Lactic acid bacteria isolated from artisanal dry sausages: characterization of antibacterial compounds and study of the factors affecting bacteriocin production. Meat Science, 87:321–329. DOI: https://doi.org/10.1016/j.meatsci.2010.11.006.

Cebeci, A.; Gurakan, C. 2003. Properties of potential probiotic Lactobacillus plantarum strains. International Journal of Food Microbiology, 20:511–518. DOI: https://doi.org/10.1016/S0740-0020(02)00174-0.

Charteris, W. P.; Kelly, P. M.; Morelli, L.; Collins, J. K. 1998a. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. Journal of Food Protection, 61:1636–1643. DOI: https://doi.org/10.4315/0362-028x-61.12.1636.

Charteris, W. P.; Kelly, P. M.; Morelli, L.; Collins, J. K. 1998b. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. Journal of Applied Microbiology, 84:759–768. DOI: https://doi.org/10.1046/j.1365-2672.1998.00407.x.

Costa, H. H. S.; Souza, M. R.; Acurcio, L. B.; Cunha, A. F.; Resende, M. F. S.; Nunes, A. C. 2013. Potencial probiótico in vitro de bactérias ácido-láticas isoladas de queijo-de-minas artesanal da Serra da Canastra, MG. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 65:1858–1866. DOI: https://doi.org/10.1590/S0102-09352013000600038.

Food and agriculture organization of the united nations and world health organization. Joint FAO/WHO working group report in drafting guidelines for the evaluation of probiotics in food. Food and Agricultural Organization of the United Nations. London, Ontario, Canadá, April 30 and May 1, 2002.

Forssten, S.; Ouwehand, A.C. 2020. Dose-Response Recovery of Probiotic Strains in Simulated Gastro-Intestinal Passage. Microorganisms, 8: 112. DOI: https://doi.org/10.3390/microorganisms8010112.

Granato, D.; Branco, G. F.; Cruz, A. G.; Faria, J. A. F., Shah, N. P. 2010. Probiotic Dairy Products as Functional Foods. Comprehensive Reviews in Food Science and Food Safety, 9: 455–470. DOI: https://doi.org/10.1111/j.1541-4337.2010.00120.x.

Guo, H.; Pan, L.; Li, L.; Lu, J.; Kwok, L.; Menghe, B.; Zhang, H.; Zhang, W. 2017. Characterization of Antibiotic Resistance Genes from Lactobacillus Isolated from Traditional Dairy Products. Journal of Food Science, 82:724–730. DOI: https://doi.org/10.1111/1750-3841.13645.

Guedes Neto, L. G.; Sousa, M. R.; Nunes, A. C.; Nicoli, J. R.; Santos, W. L.M. Atividade antimicrobiana de bactérias ácido-lácticas isoladas de queijos de coalho artesanal e industrial frente a microrganismos indicadores. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 57:245–250, 2005. DOI: https://doi.org/10.1590/S0102-09352005000800017.

Hoque, M.Z.; Akter, F.; Hossain, K.M.; Rahman, M.S.M.; Billah, M.M.; Islam, K.M.D. 2010. Isolation, identification and analysis of probiotic properties of Lactobacillus spp. from selective regional yoghurts. World Journal of Dairy & Food Sciences, 5: 39–46.

Minelli, E.B.; Benini, A. 2008. Relationship between number of bacteria and their probiotic effects. Microbial Ecology in Health and Disease, 20:180-183. DOI: https://doi.org/10.1080/08910600802408095.

Maragkoudakis, P.A.; Zoumpopoulou, G.; Miaris, C.; Kalantzopoulos, G.; Pot, B.; Tsakalidou, E. 2006. Probiotic potential of Lactobacillus strains isolated from dairy products. International Dairy Journal,16:189–199. DOI: https://doi.org/10.1016/j.idairyj.2005.02.009.

Mohanty, D.; Saini, M. R.; Mohapatra, S. 2017. In vitro study on the release of bioactive antimicrobial compounds from dairy products by certain promising probiotic *Lactobacillus* strains. International Journal of Pharmacy and Pharmaceutical Sciencies, 9:27-31. DOI: http://dx.doi.org/10.22159/ijpps.2017v9i4.12006.

Panesar, P. 2011. Fermented Dairy Products: Starter Cultures and Potential Nutritional Benefits, Food Science & Nutrition, 2:47–51. DOI: http://dx.doi.org/10.4236/fns.2011.21006.

Santos, T. T.; Ornellas, R. M. S., Acurcio, L. B.; Oliveira, M. M.; Nicoli, J. R.; Dias, C. V.; Uetanabaro, A.P.T.; Vinderola, G. 2016. Characterization of lactobacilli strains derived from cocoa fermentation in the south of Bahia for the development of probiotic cultures. LWT, 73: 259–266. DOI: https://doi.org/10.1016/j.lwt.2016.06.003.

Sharifi-Rad, J.; Rodrigues, C.F.; Stojanović-Radić, Z.; Dimitrijević, M.; Aleksić, A.; Neffe-Skocińska, K.; Zielińska, D.; Kołożyn-Krajewska, D.; Salehi, B.; Milton Prabu, S.; Schutz, F.; Docea, A.O.; Martins, N.; Calina, D. 2020. Probiotics: Versatile Bioactive Components in Promoting Human Health. Medicina, 56:433. DOI: https://doi.org/10.3390/medicina56090433.

Sharma, P.; Tomar, S.K.; Goswani, P.; Sangwan, V.; Singh, R. 2014. Antibiotic resistance among comercially available probiotics. Food Research International, 57:176–195.

Silva, B. C.; Jung, L. R. C.; Sandes, S. H. C.; Alvim, B. L.; Bomfim, M. R. Q.; Nicoli, J. R.; Neumann, E.; Nunes, A. C. 2013. In vitro assessment of functional properties of lactic acid bacteria isolated from fecal microbiota of healthy dogs for potential use as probiotics. Beneficial Microbes, 4:267-275. DOI: https://doi.org/10.3390/10.3920/BM2012.0048.

Teuber, M.; Meile, L.; Schwarz, F. , 1999.Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Van Leeuwenhoek., 76:115–137.

Vinderola, C.G.; Reinhemer, J.A. Lactic acid starter and probiotic bacteria: a comparative “in vitro” study of probiotic characteristics and biological barrier resistance. Food Research International, 36:895-904, 2003. DOI: https://doi.org/10.1016/S0963-9969(03)00098-X.