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Communication

[*Comunicação*]

In vitro evaluation of probiotic and technological properties of lactic acid bacteria isolated from artisanal cheese produced in the Serra Geral – Minas Gerais - Brazil

[*Avaliação* in vitro *de propriedades probióticas e tecnológicas de bactérias ácido-láticas isoladas de queijo artesanal da Serra Geral (MG)*]

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There is great technological potential for the use of lactic acid bacteria (LAB) isolated from artisanal dairy products, such as cheese, and it has generated an increasing interest in the study and identification of these microorganisms, in addition to the evaluation of their potential probiotic properties. Andrade *et al.* (2014) and Silva *et al.* (2019) described in vitro probiotic potential of LAB isolated from Minas artisanal cheese produced in the Canastra and Araxá regions, respectively. However, to date, there is no research in the literature on the in vitro probiotic potential of LAB isolated from artisanal cheeses produced in the Serra Geral region, as well as their raw materials and production environment. Thus, the present study aimed to evaluate the in vitro probiotic potential of LAB isolated from those cheeses, raw milk, endogenous starter cultures and swabs from surfaces. The LAB used (Table 1) were previously isolated and identified by Caldeira (2021).

The antibiogram was performed in duplicate, with three repetitions, using the disk diffusion method, according to the technique adapted from Charteris *et al.* (1998). LAB were activated twice in MRS broth (Merck, Darmstadt, Germany) and transferred to a test tube containing 3.5mL of sterile saline (0.9% NaCl) until reaching the concentration of 0.5 on the McFarland scale (10^8 CFU). Afterwards, with the aid of a sterile swab, the solution was spread on MRS agar. Twelve discs of antimicrobials (Oxoid, Basingstoke, England) were spread over the agar. The antimicrobials selected were Gentamicin (10µg); Amikacin (30µg); Penicillin G (10µg); Oxacillin (1µg); Erythromycin (15µg); Tetracycline (30µg); Chloramphenicol (30µg); Ciprofloxacin (5µg); Vancomycin (30µg); Clindamycin (2 µg); Ceftazidime (30µg) and Sulfazotrim (25µg). The plates were incubated under aerobic conditions for 48 h at 37ºC. Inhibition halos were measured using a digital caliper (Mitutoyo Sul Americana Ltda., São Paulo, Brazil) and the LAB were classified as sensitive, moderately sensitive, and resistant according to the values proposed by Charteris *et al.* (1998). The control of the test was performed using *Escherichia coli* (ATCC 25922).

The spot-on-the-law antagonism was performed in duplicate, with three repetitions, using the technique proposed by Tagg *et al.* (1976). As revealing microorganisms, four pathogens were used, namely: *Staphylococcus aureus* (ATCC 33591), *Salmonella* Typhimurium (ATCC 14028), *Listeria monocytogenes* (ATTC 15313) and *Escherichia coli* (ATTC 25922); in addition to a LAB (*Levilactobacillus brevis*), isolated by Valente (2021), from Minas artisanal cheese. For this analysis, the tested LAB were activated twice in MRS broth, incubated at 37°C for 24 h under aerobic conditions. Five µL of the activated culture were placed in the center of Petri dishes containing MRS agar. These plates were incubated at 37ºC for 48h, under aerobic conditions, forming the spots. Afterwards, 1 mL of chloroform was added to the lids of the plates, which were also exposed to ultraviolet light for

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30min. Then, 10mL of soft BHI agar (Difco Laboratories Inc., Detroit, United States) containing activated revealing microorganisms were poured over the agar containing the spot, forming a second layer. The plates were incubated at 37ºC for 48h under aerobic conditions and the inhibition halos were measured with the aid of a digital caliper.

Table 1. Lactic acid bacteria isolated from the Serra Geral artisanal cheese, its raw materials and production environment, selected for in vitro assessments of probiotic potential

Source	Code
Cheese ripened for 14 days	
Leuconostoc pseudomesenteroides	LPM
Cheese ripened for 21 days	
Lacticaseibacillus rhamnosus	LR
Levilactobacillus brevis	LB
Pediococcus pentosaceus	PP
Lactiplantibacillus plantarum	LP
Surface of benches	
Leuconostoc mesenteroides S9Z	LM
Raw milk	
Lactococcus lactis	LLSG
Endogenous starter culture	
Lactococcus lactis	LLL

The artificial gastric acid resistance was performed in duplicate, with three repetitions, using the adapted technique described by Silva *et al.* (2013). LAB were activated twice in MRS broth, which was incubated at 37°C for 24 h under aerobic conditions. Afterwards, they were centrifuged at 13,000G for 5min. The supernatant was discarded, and the pellets were washed with saline three times. Then, they were suspended in 1 mL of acid solution (hydrochloric acid, pepsin, and distilled water), pH 2.0 - for the experimental group and 1mL of saline solution (NaCl 0.9%), pH 7.0 – for group control. The samples were incubated at 37ºC for 2 h. The bacteria were centrifuged again 13,000G/5 min and washed with 0.9% saline solution to remove the acid solution, and then the pellets were suspended in MRS broth. A 2% (v/v) inoculum was also prepared in MRS broth. Then, 200µl of each sample from the control and experimental groups were transferred to a 96 well-microplate, which was incubated for 24 h in spectrophotometer equipment (Microplate, Spectrophotometer System 47 SpectraMax 340 – Molecular Devices, Sunnyvale, California, United States) at 37ºC. The absorbance of the material was determined by measuring the optical density (OD) at 620nm every hour, for 24 h, to observe inhibition. Inhibition percentage was calculated according to the formula: (1-SG/AT) x 100, in which SG and AT correspond to the areas under the growth curve of the bacteria treated with artificial gastric acid and the control, respectively. The interpretation of the results was performed based on the criteria proposed by Acurcio *et al.* (2014). The LAB were considered tolerant when they presented a percentage of inhibition below 40%, moderately tolerant from 40 to 80% and sensitive higher than 80%.

The bile salt resistance was performed similarly to the former test, except that there was no previous incubation of the samples. A volume of 200 µL of the samples treated with 0.3% Oxgall (bile salts) and MRS (control) were placed in the 96-well microplate. Absorbance was determined by measuring the OD 620nm every hour for 24 h, and the percentage of inhibition was calculated using the formula (1-SB/CT) x 100, in which SB and CT correspond to the area under the growth curve of the bacteria treated with bile salts and the control, respectively. The resistance levels were the same described for the former test.

After carrying out the in vitro tests, two BAL that obtained the best results were selected for the elaboration of fermented milks, which were submitted to physical-chemical and microbiological analyses. For this, three different batches of Molico (Nestlé®, Araçatuba, São Paulo, Brazil) skimmed milk powder were used. They were reconstituted with distilled water and sterilized at 110ºC for 10 min.

Firstly, the safety of reconstituted milk powder was tested by counts of aerobic mesophilic microorganisms (Compendium…, 2002), *Staphylococcus* spp. (Compendium…, 2002) and molds and yeasts (Tournas *et al*., 2001). The search for viable *Salmonella* spp. (Compendium…, 2002) was also carried out.

The selected BAL were evaluated, individually or in association (LLSG + LB), for their fermentative capacity, based on the evaluation of milk coagulation. For this, they were activated twice in MRS broth, which was incubated at

37°C for 24 h under aerobic conditions. After this procedure, 0.25mL of each individual LAB and 0.125mL of each mixed culture were transferred to test tubes containing 10 mL of reconstituted and sterilized powdered milk. They were incubated at 37ºC for 24 h. After that, coagulation was evaluated. Only LAB capable of fermenting milk and producing a firm clot were selected to produce fermented milks.

The fermented milk with the selected LAB was prepared using skimmed milk powder (Molico, Nestlé®, Araçatuba, São Paulo, Brazil) from three different batches, consisting of three repetitions. The skimmed milk powder was reconstituted at 10% in distilled water and was subjected to heat treatment at 110ºC for 10 minutes. They were stored at 8ºC and analyzed after 1, 7 and 14 days and submitted to physical chemical analyzes: determinations of pH (Gehaka PG1800, São Paulo, Brazil), titratable acidity (Official…, 2019); and contents of moisture, protein, and fat (Official…, 2019). The counting of BAL present in the initial inoculums and in fermented milk was also determined at 1, 7 and 14 days of storage, using serial decimal dilutions in saline solution (0.9% NaCl). Afterwards, two 10µL microdrops of the dilutions were placed on MRS agar. The plates were incubated under aerobic conditions at 37°C for 48 h. The counts of microorganisms were made by multiplying the mean number of colonies formed in the two drops by 100 and by the inverse of the dilution (Yogurt…, 1988).

Graphpad Prism 7.0 software (Graphpad Software, San Diego, California, USA) was used to perform the statistical analyses. The means of the inhibition halos of the spot-on-the-lawn antagonism were submitted to the analysis of normality by the Shapiro-Wilk test and analysis of variance by the Two-way ANOVA test. The comparison between the means was performed using the Kruskall-Wällis test with a significance level of 5%. The physicochemical parameters of the fermented milks were subjected to normality analysis using the Shapiro-Wilk test, the Two-way ANOVA and means were compared using the Tukey test, with a significance level of 5%. For bacterial count evaluations, the same statistical analysis was used after logarithmic transformation of the data.

The results of the susceptibility of the tested LAB to antimicrobials of clinical importance are shown in Table 2.

Table 2. Sensitivity profile of LAB, isolated from artisanal cheese produced in the Serra Geral region to antimicrobials of clinical importance

Drug (µg)	LMP	LR	LB	PP	LP	LM	LLSG	LLL
GEN (10)	R	R	S	R	R	R	S	R
AMI (30)	R	R	R	R	R	R	R	R
PEN (10)	S	S	S	S	S	S	S	S
OXA (1)	R	R	R	R	R	R	S	R
ERI (15)	S	S	S	S	S	S	S	S
TET (30)	S	S	S	S	S	S	S	S
CLO (30)	S	S	S	S	S	S	S	S
CIP (5)	R	MS	R	R	R	R	MS	R
VAN (30)	R	R	R	R	R	R	S	R
CLI (2)	S	S	R	S	MS	S	S	S
CAS (30)	S	MS	S	S	S	S	S	S
SUT (25)	R	R	S	R	MS	R	R	R
Total of resistance	50% (6/12)	41.6% (5/12)	41.6% (5/12)	50% (6/12)	41.6% (5/12)	50% (6/12)	16.6% (2/12)	50% (6/12)

Caption: Gentamicin = GEN; Amikacin = AMI; Penicillin = PEN; Oxacillin = OXA; Erythromycin = ERI; Tetracycline = TET; Chloramphenicol = CLO; Ciprofloxacin = CIP; Vancomycin = VAN; Clindamycin = CLI; Ceftazidime = CAS; Sulfazotrim = SUT. LPM - *Leuconostoc pseudomesenteroides*; LM – *Leuconostoc mesenteroides*; LP - *Lactiplantibacillus plantarum*; LR - *Lacticaseibacillus rhamnosus*; LB - *Levilactobacillus brevis* PP - *Pediococcus pentosaceus*; LLSG and LLL - *Lactococcus lactis*. Resistant – R; moderately sensitive – MS; sensitive –S.

It was possible to observe that all evaluated LAB were sensitive to penicillin, tetracycline, and chloramphenicol. Similar results were found by Valente *et al.* (2019) when evaluating the sensitivity profile of *Lactobacillus* to antimicrobials. Susceptibility to chloramphenicol was already expected, as its use in livestock is prohibited, due to the side effects on the bone marrow of the humans (Stival *et al.*, 2021).

According to the World Health Organization (WHO), antibiotic resistance is rising to dangerously high levels in all parts of the world as new resistance mechanisms are emerging and spreading globally. With antibiotics becoming less effective, it has grown increasingly difficult to treat patients for even common infectious diseases like pneumonia (Antibiotic…, 2020).

A total of 87.5% of the LAB showed resistance to vancomycin. Similar results were found in other studies (Costa *et al.*, 2013; Andrade, *et al.*, 2014; Valente *et al.*, 2019) with LAB isolated from Minas artisanal cheeses. Most LAB (87.5%) showed resistance to oxacillin. The same was reported by Silva *et al.* (2019), when evaluating the probiotic potential of *Lactobacillus* spp. isolated from Minas artisanal cheeses from the Araxá region.

Most of the LAB (75%) showed resistance to ciprofloxacin, which can be justified due to intrinsic characteristics of these microorganisms, related to cell wall structure, membrane permeability and efflux mechanisms (Abriouel *et al.*, 2015). These results agree with those obtained by Costa *et al.* (2013) and Andrade *et al.* (2014).

A total of 75% of the LAB showed resistance to gentamicin and all LAB were resistant to amikacin, possibly due to mechanisms related to membrane permeability or absence of cytochromes that would allow the binding of antimicrobials (Abriouel *et al.*, 2015).

From the results presented in Figure 1, it was possible to observe that all samples were able to produce inhibition halos against the tested pathogens. In addition, no LAB produced halos against the LAB isolated from Minas artisanal cheese, which is desirable for LAB screening.

Different results were found by Costa *et al.* (2013) and Valente *et al.* (2019), who described tolerant LAB isolated from Minas artisanal cheeses to acid environment. Thus, the selected LAB in the present study should be encapsulated, to survive the acid of human stomach and reach the intestine in concentrations needed to show desirable effects in the host's gastrointestinal tract.

In addition, selected potential probiotics can be conveyed into a dairy derivative since food products, such as dairy, can contribute to the survival of probiotics in gastric juice, mainly due to their buffering and protective effect (Silva *et al.*, 2021).

Regarding bile salt resistance, 87.5% of the LAB (7/8) were tolerant, only one was moderately tolerant and none were sensitive to this adverse condition. Possibly due to the development of defense mechanisms, such as the deconjugation of bile salts by the enzyme bile salt hydrolase (BSH) (Peres *et al.*, 2014). These findings differ from the results found by some researchers, in which most of the potential probiotic LAB proved to be sensitive or moderately tolerant to bile salts (Costa *et al.*, 2013; Valente *et al.*, 2019).

Lactococcus lactis (LLSG) and *Levilactobacillus brevis* (LB) were selected to produce fermented milk, as they showed the best results in vitro (Table 4). In addition, *Lactococcus lactis* is the main LAB species used as a starter culture for several foods, being commonly used by industries for fermented dairy products manufacture, such as cheeses. This culture plays a key role in relation to the quality of these products, their shelf life, preservation, and sensory attributes (Pereira *et al.*, 2020).

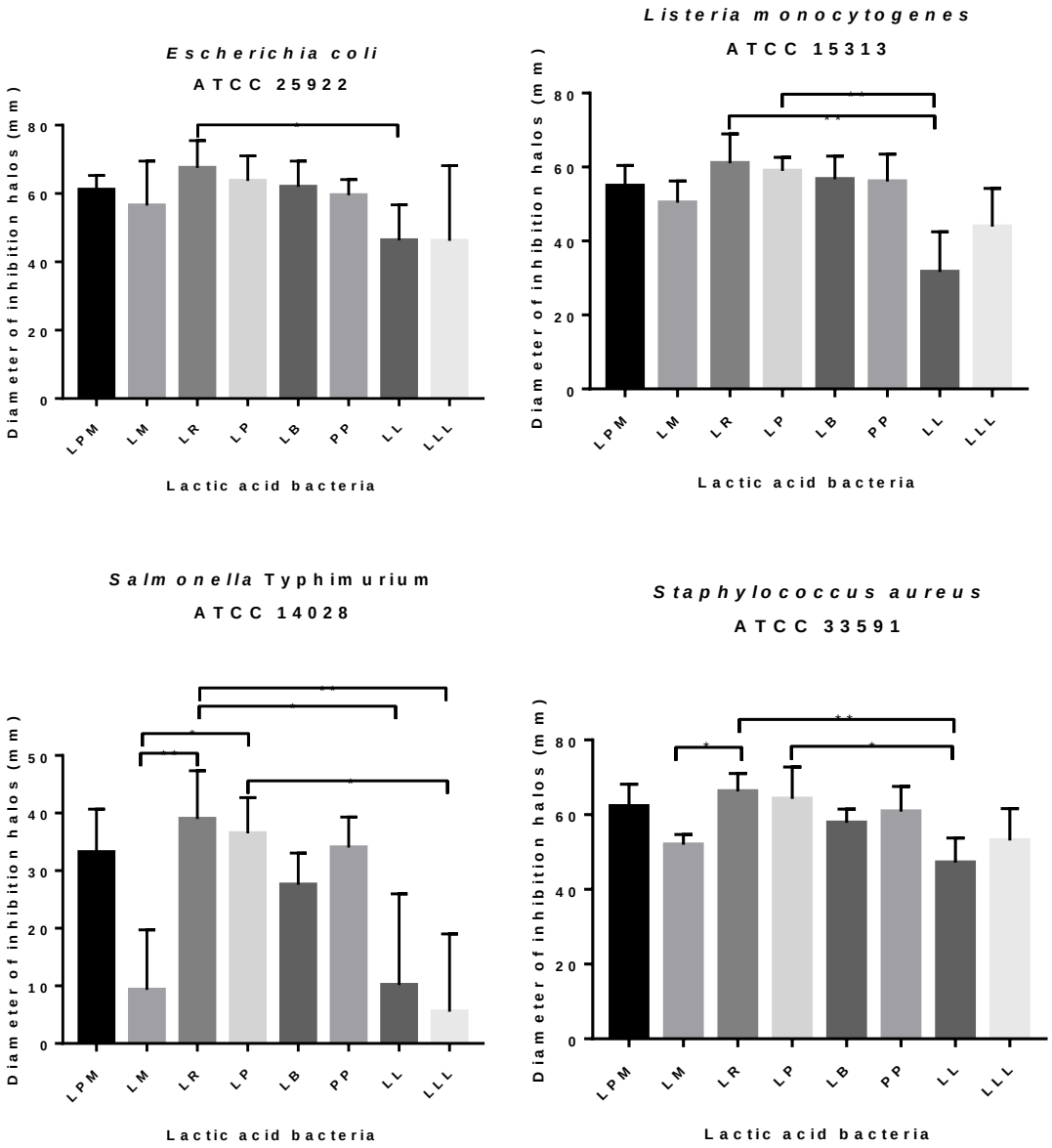

Figure 1. Mean inhibition halos (mm) and standard deviation of in vitro antagonism of LAB isolated from artisanal cheese produced in the Serra Geral region, against selected microorganisms.

Caption: LPM - *Leuconostoc pseudomesenteroides*; LM – *Leuconostoc mesenteroides*; LP – *Lactiplantibacillus plantarum*; LR - *Lacticaseibacillus rhamnosus*; LB - *Levilactobacillus brevis*. PP - *Pediococcus pentosaceus*; LLSG and LLL - *Lactococcus lactis*. Bars followed by * and ** indicate differences by the Kruskal-Wällis test ($p<0.05$ and $p<0.01$, respectively) and Dunn's post-test.

Table 3. Percentage of inhibition and level of resistance to artificial gastric acid (pH 2.0) and bile salts (Oxgall 0.3%) of LAB isolated from artisanal cheese produced in the Serra Geral region

LAB	Inhibition (%)			
	Gastric acid	Gastric acid sensivity	Bile salts	Bile salts sensivity
LPM	75.31	MT	11.00%	T
LR	85.51%	S	67.30%	MT
LB	62.27%	MT	3.70%	T
PP	75.00%	MT	29.80%	T
LP	78.50%	MT	6.08%	T
LM	82.10%	S	18.10%	T
LLSG	74.54%	MT	13.40%	T
LLL	79.00%	MT	3.80%	T

Caption: LAB = lactic acid bactéria; LPM - *Leuconostoc pseudomesenteroides*; LM – *Leuconostoc mesenteroides*; LP – *Lactiplantibacillus plantarum*; LR - *Lacticaseibacillus rhamnosus*; LB - *Levilactobacillus brevis* PP - *Pediococcus pentosaceus*; LLSG and LLL - *Lactococcus lactis*.
Tolerant (T) <40%; moderately tolerant (MT) - from 40 to 80%; sensitive (S) - higher than 80%.

Table 4. Summary of the results obtained by *Lactococcus lactis* (LLDG) and *Levilactobacillus brevis* (LB) after in vitro tests to evaluate the probiotic potential

	Tests			
LAB	Antibiogram	Antagonism	Acid resitance	Bile salts
LLSG	Sensitivity to 10 antimicrobials	Inhibition against pathogens without inhibiting desirable LAB	Moderately tolerant	Tolerant
LB	Sensitivity to 10 antimicrobials	Inhibition against pathogens without inhibiting desirable LAB	Moderately tolerant	Tolerant

After the fermentation test, only milk inoculated with LLSG and association of LLSG and LB produced a firm clot. As a result, only LLSG was selected for the elaboration of fermented milk.

The search for pathogens in reconstituted milk powder showed the absence of *Salmonella* spp. and undetectable counts of coliforms (NMP/mL), *Staphylococcus* spp., molds, and yeasts, demonstrating that the product was adequate for the elaboration of fermented milks. The inoculum used to prepare fermented milk had a count of $2x10^8$ CFU/g LAB. LAB counts in fermented milks remained high (Fig. 2) and in accordance with current legislation (Brasil, 2007) throughout the storage period.

Regarding the physicochemical analysis performed on milk fermented with LLSG at 1, 7 and 14 days of storage at 7ºC (Fig. 3), it was possible to observe a gradual increase in its acidity throughout the storage period. The acidity values were according to the current legislation (Brasil, 2007). With the increase in acidity, a decrease in the pH of the medium was expected. Despite this, it was possible to observe a gradual increase in this pH. In contrast to the production of acids, it is possible that the formation of alkaline compounds had occurred, probably on a larger scale, causing the pH to increase.

The most likely hypothesis for this formation of alkaline compounds is due to proteolysis, related to the action of proteases present in the milk or produced by LAB (Freire *et al.*, 2021). This finding supports this hypothesis since reduction in the protein content was observed during the storage period. The protein content was slightly below the minimum recommended by the legislation (Brasil, 2007) on days 1 and 14 of storage.

High moisture content was observed in the fermented milk, varying from 90.98 to 89.71 during storage. In relation to the fat content, undetectable values were observed during the

entire period of storage since skimmed milk powder was used for the preparation of fermented milk. This value is in accordance with the standards established in the Brazilian legislation (Brasil, 2007).

From the results presented, it is suggested that LLSG is suitable to be used as a potential probiotic to obtain fermented milk. However, it is necessary to carry out in vivo screening tests for its future use as a probiotic and elaboration of functional foods. Also, the present study showed the presence of beneficial bacteria in the microbiota of artisanal cheeses produced in the Serra Geral region.

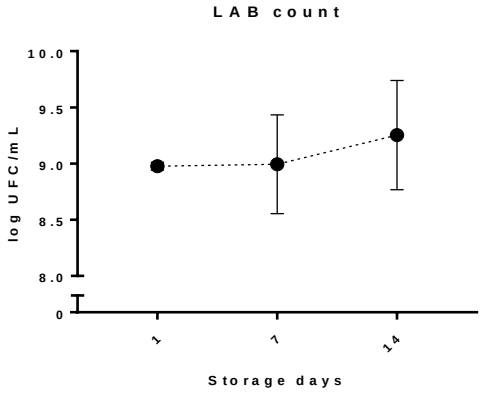

Figure 2. LAB count in fermented milk at 1, 7 and 14 days of storage at 8ºC.

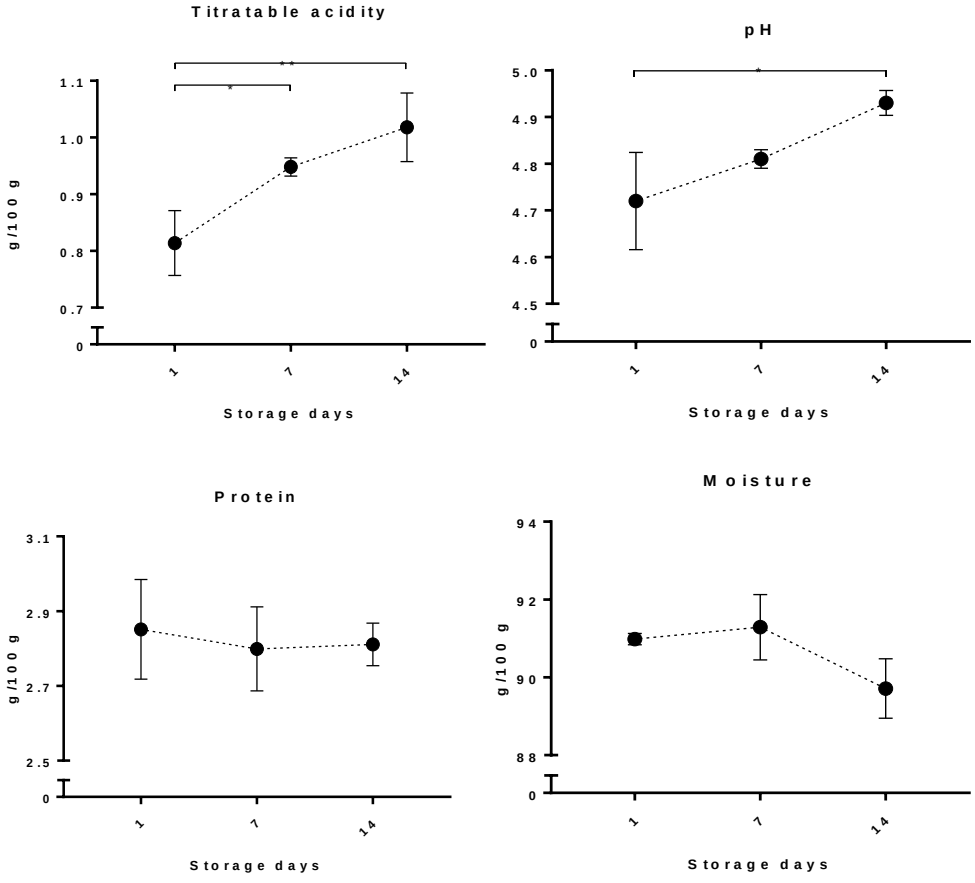

Figure 3. Physicochemical results of titratable acidity, pH and contents of protein and moisture in milk fermented by *Lactococcus lactis* (LLSG), isolated from artisanal cheese from the Serra Geral region, at 1, 7 and 14 days of storage at 8ºC.

Keywords: functional foods, raw milk cheese, Lactococcus, antibiogram, microbial antagonism

RESUMO

O presente estudo avaliou o potencial probiótico in vitro *de bactérias ácido-láticas (BAL) isoladas de queijo artesanal da Serra Geral (MG), com 14 e 21 dias de maturação, leite cru e pingo, utilizados em sua elaboração, e de bancadas de produção. As bactérias foram submetidas aos testes de antibiograma, à tolerância a ácido gástrico artificial e a sais biliares e ao antagonismo contra micro-organismos indicadores.* Levilactobacillus brevis *(Q521) e* Lactococcus lactis *(LLSG) apresentaram os melhores resultados e foram selecionados para a produção de leite fermentado. Apenas LLSG foi capaz de fermentar o leite. O produto apresentou qualidade microbiológica e físico-química adequada, exceto para os teores de proteína, segundo a legislação vigente para leites fermentados, demonstrando potencial tecnológico. A partir dos resultados apresentados, sugere-se que testes* in vivo *sejam realizados para que LLSG possa vir a ser utilizado como probiótico.*

Palavras-chave: alimentos funcionais, queijos de leite cru, Lactococcus*, antibiograma, antagonismo microbiano*

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