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In vitro assessment of the probiotic potential of lactobacilli isolated from Minas artisanal cheese produced in the Araxá region, Minas Gerais state, Brazil

[*Avaliação in vitro do potencial probiótico de lactobacilos isolados de queijo minas artesanal produzido na região de Araxá, estado de Minas Gerais, Brasil*]

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ABSTRACT

Minas artisanal cheese is made from endogenous starter cultures, including lactic acid bacteria (LAB). Some LAB may possess probiotic potential. Thus, this study aimed to evaluate the *in vitro* probiotic properties of lactobacilli isolated from Minas artisanal cheeses produced in Minas Gerais. Ten samples of lactobacilli, formerly isolated from those cheeses, were submitted to the following assays: antimicrobial susceptibility, tolerance to artificial gastric juice and biliary salts, production of hydrogen peroxide and antagonism against pathogenic and non-pathogenic micro-organisms. Only *L. plantarum* (C0) was sensitive to all tested antimicrobials, while the other LAB samples were resistant to at least one drug. Six samples were tolerant to artificial gastric juice, and *L. brevis* (A6) even grew in that medium. Three samples were tolerant to biliary salts. Only *L. brevis* (E35) produced hydrogen peroxide. Difference ($P< 0.05$) was observed among the means of inhibition haloes of lactobacilli against *Enterococcus faecalis* ATCC 19433 and *Lactobacillus plantarum* C24 in spot-on-the-lawn assay. All samples of lactobacilli inhibited *Escherichia coli* ATCC 25922, *Salmonella enterica* var. Typhimurium ATCC 14028 in co-culture antagonism test ($P< 0.0001$). Most lactobacilli samples showed *in vitro* probiotic potential. From the tested samples, *L. brevis* (A6) presented the best results considering all in vitro probiotic tests.

Keywords: artisanal cheeses, beneficial micro-organisms, antibiogram, tolerance to gastric juice and biliary salts, antagonism

RESUMO

O queijo minas artesanal é produzido por culturas starters *endógenas, incluindo bactérias ácido-láticas (BAL). Algumas BAL podem possuir potencial probiótico. Com isso, este estudo teve como objetivo avaliar as propriedades probióticas* in vitro *de lactobacilos isolados de queijo minas artesanal produzido no estado de Minas Gerais. Dez amostras de lactobacilos, previamente isoladas desses queijos, foram submetidas aos seguintes testes: susceptibilidade aos antimicrobianos, tolerância ao suco gástrico artificial e aos sais biliares, produção de peróxido de hidrogênio e antagonismo contra micro-organismos patogênicos e não patogênicos. Apenas* L. plantarum *(C0) foi sensível a todos os antimicrobianos testados, enquanto as outras amostras de BAL foram resistentes a, pelo menos, uma droga testada. Seis amostras foram tolerantes ao suco gástrico artificial, e* L. brevis *(A6) apresentou crescimento nesse meio. Três amostras foram tolerantes aos sais biliares. Apenas* L. brevis *(E35) produziu peróxido de hidrogênio. Diferença ($P<0,05$) foi observada entre as médias dos halos de inibição de lactobacilos contra* Enterococcus faecalis *ATCC 19433 e* Lactobacillus plantarum *C24 no teste do* spot-on-the-lawn. *Todas as amostras de lactobacilos inibiram* Escherichia coli *ATCC 25922,* Salmonella enterica *var. Typhimurium ATCC 14028 no teste de antagonismo em cocultura ($P<0,0001$). A maioria das amostras de lactobacilos apresentou potencial probiótico* in vitro. *Com base nas amostras testadas,* L. brevis *(A6) apresentou os melhores resultados, considerando-se todos os testes probióticos* in vitro.

Palavras-chave: queijo artesanal, micro-organismos benéficos, antibiograma, tolerância ao suco gástrico e sais biliares, antagonismo

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INTRODUCTION

Minas artisanal cheese has long been produced in some regions of Minas Gerais State, Brazil, involving a large number of small farmers, generating labor and income to their families. Its production is characterized by the use of raw milk and endogenous starter cultures and the cheeses are ripened at farms at room temperature (Dores and Ferreira, 2012).

Lactic acid bacteria (LAB) present in raw milk, endogenous cultures and the environment of the cheesemaking facilities are responsible to the fermentation and the acidifying processes that occur during artisanal cheese production, mainly during the ripening. The metabolism of this microbiota confers to the cheeses the unique characteristics of flavor and texture (Kongo *et al*., 2007; Dolci *et al*., 2008).

According to a joint of experts from the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Organização…, 2002).

LAB isolated from artisanal cheeses may possess probiotic potential, which have been investigated in vitro (Mandal *et al*., 2016). From the seven regions officially recognized by the Minas Gerais State government as artisanal cheese producers (Araxá, Serra da Canastra, Cerrado, Campo das Vertentes, Serro, Serra do Salitre and Triângulo), potentially probiotic LAB have been isolated from chesses produced in the Serra da Canastra region (Costa *et al*., 2013; Andrade *et al*., 2014). However, there is a lack of information concerning the probiotic potential of LAB present in Minas artisanal cheeses from the Araxá region.

Thus, this study aimed at the in vitro screening of potentially probiotic lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region, Minas Gerais State.

MATERIAL AND METHODS

Ten samples of lactobacilli were previously isolated from 84 samples of Minas artisanal cheese produced in the Araxá region and were identified by 16S rRNA gene sequencing according to Reysenbach *et al*. (2000). The samples *L. brevis* (A6, B16 and E35), *L. casei* (B5), *L. plantarum* (B206, C0, D4 and E5) and *L. rhamnosus* (A1 and C5) were submitted to the investigation of in vitro probiotic potential. All tests were carried out in triplicate with two repetitions each.

The antimicrobial susceptibility test was performed by the agar disk diffusion method according to Charteris *et al*. (1998). The isolated lactobacilli were grown on MRS agar (Oxoid, Basingstoke, England), under aerobiosis, for 24–48h at 37ºC. From their colonies, concentrations of 10^8 viable cells (0,5 McFarland scale) were prepared using 3.5ml of 0.85% buffered saline. Swabs from those dilutions were spread onto the surface of 14cm diameter plates containing MRS agar (Oxoid). The drug disks (Oxoid) were distributed on the surface of the plates, which were incubated under aerobiosis, for 24–48h at 37ºC. Then, the diameters of the inhibition zones were determined using a digital pachymeter. The antimicrobials (Oxoid®, Basingstoke, England) were: ceftazidime - CAZ (30μg), ciprofloxacin - CIP (5μg), clindamycin - DA (2μg), erythromycin - E (15μg), streptomycin – S (30μg), gentamicin - GN (10μg), oxacillin - OX (1μg), penicillin - PEN (10UI), tetracycline - TE (30μg) and vancomycin - VA (30μg). Quality control of discs containing the antimicrobials was performed using *Escherichia coli* ATCC 25922. Lactobacilli samples were classified as resistant, moderately sensitive and sensitive to the drugs (Charteris *et al*., 1998).

The tolerance to artificial gastric juice was carried out according to Neumann (1991) and Silva *et al*. (2013). Lactobacilli samples were cultured in MRS broth (Difco Laboratories Inc., Detroit, USA) for 24h at 37ºC, under aerobiosis. After growth, 1 mL of each culture was transferred to *Eppendorf*® microtubes and diluted 10X in 0.9% saline, pH 7.0 (control) and in artificial gastric juice (pepsin 3g/L, pH 2.0). After that, 200uL were transferred to the wells of 96-well microplate and incubated in a spectrophotometer (Microplate Spectrophotometer System 47 SpectraMax 340 - Molecular Devices, Sunnyvale, USA) for 12h at 37ºC. The absorbance was determined by the OD_{620nm} measured each 30min and the growth

inhibition percentage was calculated using the program Graphpad Prism 5.0 by the formula (1-SG/CT) x 100. SG and CT correspond to the areas under the growth curve of the bacteria treated with artificial gastric juice and control, respectively. The interpretation of the results, expressed in inhibition percentage, was proposed by Acurcio *et al*. (2014), as following: tolerant <40%, moderate tolerant 40-80% and sensitive higher than 80%.

The tolerance to biliary salts was performed according to Walker and Gilliland (1993) and Silva *et al*. (2013). Lactobacilli samples were cultured in MRS broth (Difco) for 24h at 37ºC, under aerobiosis. After growth, 1mL of each culture was transferred to *Eppendorf*® microtubes, diluted 4% (v/v) in MRS broth (Difco). Then, 100µL were transferred to a well of the 96-well microplate containing 100µL of MRS broth (Difco) and 100µL to another well containing MRS broth (Difco) added with 0.6% (p/v) biliary salts (Oxgall®). The microplates were incubated in a spectrophotometer (Microplate Spectrophotometer System SpectraMax 340 - Molecular Devices, Sunnyvale, USA) for 12h at 37ºC. The absorbance was determined by the OD_{620nm} measured each 30min and the growth inhibition percentage was calculated using the program Graphpad Prism 5.0 by the formula (1-SB/CT) x 100. SB and CT correspond to the areas under the growth curve of the bacteria treated with biliary salts and control, respectively. The interpretation of the results, expressed in inhibition percentage, was proposed by Acurcio *et al*. (2014), as following: tolerant <40%, moderately tolerant 40-80% and sensitive higher than 80%.

The synthesis of hydrogen peroxide was evaluated according to the colorimetric method described by Rabe and Hillier (2003). Lactobacilli samples were cultured in MRS broth (Difco) for 24h at 37ºC, under aerobiosis. After growth, 2µL were inoculated onto TMB-plus agar (Brucella agar 43.0g; tetramethylbenzidin dihydrochloride 0.25g; starch 20.0g; hemin solution (0.05%), 10.0mL; MgSO4 0.57g; MgSO4.H2O 0.12g; peroxidase solution (0.1%), 10mL; and horse serum, 50mL, in 1 liter of distillated water). The plates were incubated for 18 hours at 37°C in anaerobiosis, and then exposed to air for 30min. Colonies of lactobacilli were considered producers of hydrogen peroxide when showed bluish or brownish shades due to the oxidative activity. They were considered of high production (+ + +) for brown colonies, mean producer (+ +) for blue colonies, low producer (+) for white-blue colonies and absence of production (-).

The antagonism of the lactobacilli samples against indicator bacteria was carried out by the spot-on-the-lawn assay, described by Tagg *et al*. (1976). The isolated bacteria were cultured in MRS broth (Difco) for 24h at 37ºC under aerobiosis. After growth, an aliquot (5µL) of the culture was spotted onto MRS agar (Difco). After incubation at 37ºC for 48h under aerobiosis, the cells were killed by exposure to chloroform during 20min. Residual chloroform was allowed to evaporate and Petri dishes were overlaid with 3.5ml of BHI or MRS soft (0.7%) agar (Difco) which had been inoculated with 10µL of a 24h culture of *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica* var. Typhimurium ATCC 14028, *Shigella flexneri* ATCC 25875 and *Staphylococcus aureus* N315. *L. plantarum* C24 and *L. rhamnosus* A23, isolated from the same cheeses and molecularly identified by 16S RNA gene sequencing, were also used as indicator strains. After 24h of incubation at 37ºC, under aerobiosis, the plates were evaluated for the presence of a growth inhibition halo. Mean values of the inhibition haloes were compared by the Kruskal-Wallis test at 5% significance.

The direct antagonism test (co-culture) was performed according to the method described by Hutt *et al*. (2006). Lactobacilli were tested against *Escherichia coli* ATCC 25922, *Salmonella enterica* var. Typhimurium ATCC 14028 and *Shigella flexneri* ATCC 25875. The tested bacteria were cultured in MRS (LAB) or BHI (Gram negative bacteria) broths (Difco) for 24h at 37ºC under aerobiosis. After growth, 1mL of each culture was inoculated into MRS (Difco) and BHI (Difco) broths, at 1% dilution (v/v), inside *Eppendorf*® microtubes, which were incubated at 37ºC for 18h. Then, serial dilutions of each culture were prepared using 0.9% saline and 10µL of them were spread on MacConkey agar (Difco). The plates were incubated for 24 hours, at 37ºC, under aerobiosis. After that, the colonies that grew on the agar were counted. The

Friedman test at 0.0001% significance level was used to compare mean counts of bacteria. Control of pH of MRS and BHI broths after incubation under aerobiosis for 24h at 37°C was carried out using a digital pH meter.

RESULTS AND DISCUSSION

The results of antimicrobial susceptibility test (Table 1) showed that only *L. plantarum* C0 was sensitive to all tested drugs. The other samples of *Lactobacilllus* spp. isolated from Minas artisanal cheeses produced in the Araxá region were resistant to at least one drug.

The results of the present study are different from the description of other researches (Costa *et al*., 2013; Andrade *et al*., 2014) that evaluated the antimicrobial susceptibility of lactic acid bacteria (LAB) isolated from Minas artisanal cheeses produced in the Serra da Canastra region. The former studies did not report any lactobacilli sensitive to all tested drugs. According to Souza *et al*. (2007), the sensitivity to a larger number of antimicrobials, belonging to different classes, is essential to a probiotic microorganism, since it decreases the chance of introducing genes that confer resistance to antimicrobials in an ecosystem, like the gastrointestinal tract of human beings.

L. plantarum E5 showed the highest percentage of resistance to antimicrobials (60%) and may represent a risk to undesirable transmission of drug resistance genes. Multiple antimicrobials resistance in LAB isolated from cheeses is reported in the literature (Flórez *et al*., 2005). Consequently, there is a concern about the non-intrinsic resistance genes which may be passed to pathogens and cause problems to public health.

Table 1. Profile of antimicrobial susceptibility of lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region, tested by agar disk diffusion method

Sample	Antimicrobial									
	GN	OX	VA	S	TE	CAZ	PEN	DA	CIP	E
L. brevis A6	S	R	R	S	S	S	S	S	MS	S
L. brevis B16	S	R	R	R	MS	MS	MS	R	R	S
L. brevis E35	S	R	R	R	S	MS	MS	R	R	S
L. casei B5	R	R	S	R	S	R	S	S	S	R
L. plantarum B206	R	R	R	R	S	MS	S	S	MS	S
L. plantarum C0	S	S	S	S	S	S	S	S	S	S
L. plantarum D4	S	R	S	MS	MS	R	S	MS	S	S
L. plantarum E5	R	R	R	R	S	R	S	S	S	R
L. rhamnosus A1	R	R	R	R	S	R	S	S	S	S
L. rhamnosus C5	R	R	R	R	S	R	S	S	MS	S

Legend: ceftazidime (CAZ), clindamycin (DA), ciprofloxacin (CIP), erythromycin (E), gentamicin (GN), oxacillin (OX), penicillin (PEN), streptomycin (S), tetracycline (TE), vancomycin (VA). R – resistant, MS – moderately sensitive, S – sensitive, according to Charteris *et al*. (1998).

The highest sensitiveness percentages were reported to erythromycin, penicillin and tetracycline, all 80% (8/10). Regarding penicillin, the results of the present study are similar to those reported by Danielsen and Wind (2003), describing that lactobacilli are sensitive to penicillin and other antimicrobials used in human medicine. A total of 90% (9/10) of the tested lactobacilli in the present study were resistant to oxacillin. Only *L. plantarum* C0 was sensitive to this antimicrobial, which may be associated to an intrinsic resistance, as suggested by Danielsen and Wind (2003); Mathur and Singh (2005).

Enterococcus spp., *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. may show intrinsic resistance to vancomycin (Ouoba *et al*., 2008; Hegstad *et al*., 2010). However, in the present study, *L. casei* B5, *L. plantarum* C0 and *L. plantarum* D4 were sensitive to that drug, opposing to the results described by Costa *et al*. (2013) and Andrade *et al*. (2014), who observed 100% vancomycin resistance in samples of LAB isolated from Minas artisanal cheeses. On the other hand, Mannu *et al*. (2003); Herreros *et al*. (2005); Belletti *et al*. (2009); Acurcio *et al*. (2014) reported LAB sensitive to vancomycin, showing that resistance is not present in all samples of lactobacilli.

From the results, it was observed that 60% of tested LAB samples showed resistance to the evaluated antimicrobials. *L. plantarum* C0, *L. brevis* A6, *L. plantarum* B206 and *L. plantarum* D4 showed the best results, with 0, 20, 20 and 40% of antimicrobial resistance, respectively.

Most lactobacilli samples were tolerant to artificial gastric juice (Table 2), especially *L. brevis* A6. Only one sample was sensitive. Since that assay mimics what may happen inside human stomach, acid-tolerant lactobacilli would survive to the stressing environment, which is essential for them to reach the intestines, where they would present probiotic activity. Similar results were described by Costa (2013) and Andrade (2014) who showed that 100% of the lactobacilli isolated from Minas artisanal cheeses from the Serra da Canastra region were tolerant to artificial gastric juice.

Table 2. Inhibition percentage and classification according to tolerance to artificial gastric juice (pH 2.0) and to biliary salts (0.3%) of lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region

Sample	Gastric juice	Biliary salts
L. brevis A6	-0.38 (T)	36.13 (T)
L. brevis B16	78.15 (MT)	79.95 (MT)
L. brevis E35	9.42 (T)	53.31 (MT)
L. casei B5	97.00 (S)	25.40 (T)
L. plantarum B206	27.69 (T)	66.22 (MT)
L. plantarum C0	75.84 (MT)	78.21 (MT)
L. plantarum D4	34.81 (T)	64.68 (MT)
L. plantarum E5	6.39 (T)	20.69 (T)
L. rhamnosus A1	34.93 (T)	70 (MT)
L. rhamnosus C5	73.60 (MT)	61.16 (MT)

Legend: tolerant (T); moderately tolerant (MT); sensitive (S), according to criteria established by Acurcio *et al.* (2014).

Tolerance to acid is common to lactobacilli. *L. brevis* A6 showed grow even in the presence of artificial gastric juice (Table 2). According to Jay (2005), some samples of that species grow in acid medium, being able to develop at pH 3.16. According to Dunne *et al*. (1999), that tolerance is potentialized in probiotic samples.

The tolerance to acid of the studied LAB may also be explained by the origin of them, since they were isolated from cheeses which are an acid food. According to Resende *et al*. (2011), a low pH is present in all the cheese processing and this factor acts like a selective medium favoring acid tolerant bacteria to survive and dominate the microbiota.

The best results for the tolerance to artificial gastric juice were demonstrated by *L. rhamnosus* A1, *L. brevis* A6, *L. plantarum* B206, *L. plantarum* D4, *L. plantarum* E5 and *L. brevis* E35 which showed the lowest inhibition values, mainly *L. brevis* A6 that also was able to grow at the acid conditions, simulating the gastric environment.

Differently to the acid tolerance, the test of tolerance to biliary salts showed that 30% (3/10) of the lactobacilli samples were tolerant and 70% (7/10) were moderately tolerant to that condition (Table 2). None sample was considered sensitive.

The tolerance levels of lactobacilli found in this study were lower to those reported by Andrade *et al*. (2014) and higher than those values described by Costa *et al*. (2013); both analyzed in vitro probiotic potential of LAB isolated from Minas artisanal cheese from the Serra da Canastra region. Some studies have shown variation in the tolerance to biliary salts exhibited by LAB isolated from several sources including ewe milk, canine feces, among others (Silva *et al*., 2013; Acurcio *et al*., 2014). These results indicate that bacteria originated from different environments may present distinct tolerance to biliary salts.

L. plantarum E5 showed the lowest percentage of biliary salts inhibition. LAB from the same species also presented similar behavior (Costa *et al*., 2013; Andrade *et al*., 2014). However, this tolerance is variable even for bacteria from the

same species, as seen in Table 2. According to Ruiz-Moyano *et al*. (2008), tolerance to biliary salts is more dependent of the bacterial strain than of the bacterial species.

Despite of the variation of the results, none of the analyzed bacteria was considered sensitive to biliary salts, which is also desirable for the screening of probiotic samples. It is relevant to remember that biliary salts action is important to remove pathogenic bacteria from the intestines since they act like a detergent on the plasmatic membrane of the microorganisms. However, this phenomenon is not selective and also affects desirable bacteria. The mechanisms that determine the tolerance of bacteria to biliary salts are not elucidated yet. It is believed that some microorganisms produce an enzyme that hydrolyzes biliary salts avoiding their detergent action (Vinderola and Reinheimer, 2003).

L. brevis A6, *L. casei* B5 and *L. plantarum* E5 showed the best results of tolerance to biliary salts, mainly the *L. plantarum* E5 which showed the better capacity of survival in the intestine.

The production of hydrogen peroxide by lactobacilli has been pointed out as an important antimicrobial action. The substance acts against pathogens like *Salmonella* spp. (Lebeer *et al*., 2008; Predmore *et al*., 2008). Samples of peroxide-producer lactobacilli are frequently isolated from the vagina of healthy women (Servin, 2004; Martin and Suarez, 2010; Silva *et al*., 2013).

In the present study, only *L. brevis* E35 produced hydrogen peroxide (10%) and it was considered low production (+). LAB isolated from other sources, like intestinal mucous of dogs, produced hydrogen peroxide in variable amounts (8-92.8%), indicating that the microbial habitat may influence the ability in producing that substance (Silva *et al*., 2013).

Most of the lactobacilli samples showed antagonism activity against indicator microorganisms (Table 3). Some differences in the antagonism against LAB used by indicator microorganisms were detected. *E. faecalis* ATCC 19433 was more inhibited by *L. rhamnosus* A1 than by *L. plantarum* C0 and D4 ($P< 0.05$) while *L. plantarum* C24 was more inhibited by *L. rhamnosus* C5 than by *L. plantarum* D4 and *L. brevis* B16 ($P< 0.05$).

Table 3. Means of inhibition haloes (mm) lactobacilli, isolated from Minas artisanal cheeses produced in the Araxá region, against indicator microorganisms after spot-on-the-lawn assay

Sample	Indicator microorganism							
	EC	EF	LM	ST	SF	SA	LP C24	LR A23
LR A1	57.13	28.86[a]	24.57	46.68	43.52	31.11	28.20 [a. b]	0.00
LB A6	45.59	6.27[b. c]	22.68	51.92	49.00	36.96	27.80 [a. b]	0.00
LB B16	42.49	5.79 [b. c]	21.93	40.76	51.74	32.74	5.87 [c]	8.96
LC B5	56.28	16.08 [a. b. c]	4.51	25.82	25.14	0.00	20.60 [b. c]	0.00
LP B206	34.08	26.76 [a. b]	26.02	44.74	50.22	32.67	26.12 [a. b. c]	0.00
LP C0	17.11	0.00 [c]	24.70	33.72	9.82	4.21	17.91 [a. b. c]	0.00
LR C5	52.00	26.86 [a. b]	25.21	44.64	45.98	28.47	29.14 [a]	0.00
LP D4	21.63	0.00 [c]	6.72	0.00	23.80	0.00	0.00 [c]	0.00
LP E5	55.80	17.73 [a. b. c]	18.13	28.24	29.57	0.00	23.55 [a. b. c]	0.00
LB E35	45.06	5.85 [b. c]	22.73	42.80	47.64	28.87	23.15 [a. b. c]	0.00

Legend: Means followed by distinct superscript letters in the same columns are different according to Kruskal-Wallis ($P< 0.05$). EC – *Escherichia coli* ATCC 25922; EF – *Enterococcus faecalis* ATCC 19433; LM – *Listeria monocytogenes* ATCC 15313; ST – *Salmonella enterica* var. Typhimurium ATCC 14028; SF - *Shigella flexneri* ATCC 25875; SA – *Staphylococcus aureus* N315, LB - *Lactobacillus brevis*, LC - *Lactobacillus casei*, LP - *Lactobacillus plantarum*, LR - *Lactobacillus rhamnosus*.

Similar antagonistic activity of LAB isolated from Minas artisanal cheese from the Serro region, "coalho" cheese and Minas artisanal cheese from the Serra da Canastra region against indicator microorganisms were reported by Alexandre *et al*. (2002); Guedes Neto *et al*. (2005); Andrade *et al*. (2014), respectively.

The development of *S. aureus* is associated with intrinsic factors, such as the presence of vitamins

B (thiamine and nicotic acid), inorganic salts and amino acids, as well as the nitrogen source, especially arginine, cystein, proline and valine, as well a temperature between 7° and 48°C (at 37°C considered ideal) (Adams and Moss, 2008). The presence of these factors in the culture media and environment where the tests were performed may be related to the absence of inhibition of *S. aureus* N315 by *L. casei* B5 and *L. plantarum* D4 and E5. In addition, the presence of *Staphylococcus* spp. in high counts in Minas artisanal cheeses described in some studies may also be associated with inhibition observed in this study (Resende *et al*., 2011; Castro *et al*., 2016). Those undesirable bacteria may produce enterotoxins in contaminated cheeses and cause food poisoning (Schelin *et al*., 2011). Their presence in artisanal cheeses is related to the occurrence of mastitis in herds and cheese contamination by handlers who are carriers of the bacteria (Huber *et al*., 2010). Thus, is very important to adopt mastitis control in dairy herds, good manufacturing practices in cheesemaking and use antagonistic LAB to avoid staphylococcal presence in artisanal cheeses.

Probably, the antagonistic activities detected in the present study were caused by the lactic acid produced by the tested LAB. This organic acid has bacteriostatic or bactericidal effect against sensitive microorganisms (Grajek *et al*., 2005). Bacteriocins produced by LAB may also be involved in the antagonism, since they inhibit the growth of samples of *E. coli*, *E. faecalis*, *Salmonella* spp. and *S. aureus* tested by the spot-on-the-lawn assay (Jamuna *et al*., 2005; Garcia, 2006; Todorov and Dicks, 2007; Todorov, 2009). The inhibition activity showed by *L. brevis* E35 may be related to the production of hydrogen peroxide, as previously discussed. However, it was not produced in large amounts and the antagonism of the indicator bacteria may be caused by the association of the substances described in this paragraph.

LAB should inhibit only undesirable bacteria; however, they also may alter the development of other bacteria from the same group. Considering this criterion for the screening of probiotic samples, from the tested LAB, *L. plantarum* D4 would be selected for use in the production of fermented dairies. That sample showed the lowest inhibitory activity against other LAB ($P< 0.05$).

L. rhamnosus A1, *L. brevis* A6 and B16, *L. plantarum* B206, *L. rhamnosus* C5 and *L. brevis* E35 inhibited all pathogens and also should be selected for further in vivo probiotic studies and use for cheesemaking.

The results of the co-culture antagonism assay (Table 4, 5 and 6) showed that there was reduction in bacterial counts after growth in BHI and MRS broths. However, values of bacterial counts were always higher ($P< 0.0001$) after growth in BHI when compared to MRS for all co-culture antagonism against indicator pathogens (*E. coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028 and *S. flexneri* ATCC 25875), except for antagonism of *Lactobacillus plantarum* C0 against *Shigella flexneri* ATCC 25875 ($P> 0.0001$).

Table 4. Mean values of *E. coli* ATCC 25922 counts (CFU/mL) on MacConkey agar, after growth in BHI and MRS broths with or without the presence of lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region

Broth	Co-culture										
	EC	A1+EC	A6+ EC	B16+ EC	B5+ EC	B206+ EC	C0+ EC	C5+ EC	D4+ EC	E5+ EC	E35+ EC
BHI	1,18 x 10^{5a}	4,25 x $10^{9\ a}$	3,25 x $10^{9\ a}$	2.49 x 10^{9a}	5.42x 10^{9a}	7.50 x 10^{9a}	6.5x 10^{10a}	5.16x 10^{9a}	9.17x 10^{9a}	1.16x 10^{9a}	1x 10^{9b}
MRS	3,83 x 10^{5b}	< 1 x 10^{1b}	< 1 x 10^{1b}	8.83 x 10^{5b}	0.33x 10^{5b}	< 1 x 10^{1b}	3 x 10^{6b}	< 1 x 10^{1b}	1.33x 10^{5b}	1x 10^{5b}	1.78x 10^{6b}

Legend: means followed by distinct superscript letters in the column row are different according to Friedman test ($P< 0.0001$). EC: *Escherichia coli* ATCC 25922, A1: *Lactobacillus rhamnosus*, A6: *Lactobacillus brevis*, B16: *Lactobacillus brevis*, B5: *Lactobacillus casei*, B206: *Lactobacillus plantarum*, C0: *Lactobacillus plantarum*, C5: *Lactobacillus rhamnosus*, D4: *Lactobacillus plantarum*, E5: *Lactobacillus plantarum*, E35: *Lactobacillus brevis*.

Table 5. Mean values of *Salmonella* Typhimurium ATCC 14028 counts (CFU/mL) on MacConkey agar, after growth in BHI and MRS broths with or without the presence of lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region

Broth	Co-culture										
	ST	A1+ ST	A6+ ST	B16+ ST	B5+ ST	B206+ ST	C0+ ST	C5+ ST	D4+ ST	E5+ ST	E35+ ST
BHI	1.11x 10^{10a}	1.5 x 10^{10a}	6.25x 10^{9a}	1.3 x 10^{10a}	9.58x 10^{9a}	7.50 x 10^{9a}	1.17x 10^{10a}	1.39x 10^{10a}	3.59 x 10^{9a}	1.3 x 10^{10a}	7.41 x 10^{9a}
MRS	0.5 x 10^{5b}	< 1 x 10^{1b}	< 1 x 10^{1b}	< 1 x 10^{1b}	0.5x 10^{5b}	< 1 x 10^{1b}	1x 10^{5b}	< 1 x 10^{1b}	0.33 x 10^{5b}	1 x 10^{5b}	< 1x 10^{1b}

Legend: means followed by distinct superscript letters in the same column are different according to Friedman test (P< 0.0001). ST: *Salmonella* Typhimurium ATCC 14028, A1: *Lactobacillus rhamnosus*, A6: *Lactobacillus brevis*, B16: *Lactobacillus brevis*, B5: *Lactobacillus casei*, B206: *Lactobacillus plantarum*, C0: *Lactobacillus plantarum*, C5: *Lactobacillus rhamnosus*, D4: *Lactobacillus plantarum*, E5: *Lactobacillus plantarum*, E35: *Lactobacillus brevis*.

Table 6. Mean values of *Shigella flexneri* ATCC 25875 counts (CFU/mL) on MacConkey agar, after growth in BHI and MRS broths with or without the presence of lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region

Broth	Co-culture										
	SF	A1+ SF	A6+ SF	B16+ SF	B5+ SF	B206+ SF	C0+ SF	C5+ SF	D4+ SF	E5+ SF	E35+ SF
BHI	4 x 10^{9a}	9.16 x 10^{9a}	5.25 x 10^{9a}	1 x 10^{10a}	1 x 10^{10a}	5 x 10^{9a}	3.92 x10^{9}	6.58 x 10^{9a}	1.4 x 10^{9a}	5.50 x 10^{9a}	9.33 x 10^{9a}
MRS	1.66x 10^{5b}	< 1x 10^{1b}	< 1x 10^{1b}	< 1x 10^{1b}	< 1x 10^{1b}	< 1x 10^{1b}	1.50x 10^{5}	< 1x 10^{1b}	< 1x 10^{1b}	< 1x 10^{1b}	0.17x 10^{5b}

Legend: means followed by distinct superscript letters in the same column are different according to Friedman test (P< 0.0001). SF: *Shigella flexneri* ATCC 25875, A1: *Lactobacillus rhamnosus*, A6: *Lactobacillus brevis*, B16: *Lactobacillus brevis*, B5: *Lactobacillus casei*, B206: *Lactobacillus plantarum*, C0: *Lactobacillus plantarum*, C5: *Lactobacillus rhamnosus*, D4: *Lactobacillus plantarum*, E5: *Lactobacillus plantarum*, E35: *Lactobacillus brevis*.

The co-culture antagonism results indicate that the pathogenic bacteria inhibition was more accentuated when they were previously incubated in the presence of lactobacilli. It may be explained by the production of antimicrobial substances (mainly organic acids) when LAB were cultured in MRS broth together with the pathogens.

Hutt *et al*. (2006) observed antagonistic activity of probiotic samples of *Lactobacillus* spp. and *Bifidobacterium* spp. against *E. coli*, *Salmonella enterica* spp. *enterica* and *Shigella sonnei*. They suggested that the antagonism was favored in liquid medium due to the fast diffusion of antimicrobial substances, like organic acids. Garcia (2006) also suggested an association between the antagonistic activity of lactobacilli with the production of organic acids leading to a pH decline.

An accentuated decrease in pH of MRS broth after growth of all tested lactobacilli was observed. Thus, the LAB acidified the medium to a lower pH values than did the pathogens after growth in BHI broth (Table 7), demonstrating their higher potential in production of acid and survivor in a lower pH.

The pH of MRS broth after incubation of *L. rhamnosus* A1 and C5, *L. brevis* A6 and *L. plantarum* B206 was lower than control. The same LAB samples totally inhibited the three indicator pathogens, strengthening the hypothesis that the antagonistic effect was caused due to the production of organic acids. It may be stressed that *E. coli*, *S.* Typhimurium and *S. flexneri* requires minimum pH values of 4.5, 4.5 and 5.5, respectively, for growing (Jay, 2005; Cardoso and Carvalho, 2006).

Table 7. Mean values of pH of MRS and BHI broths after incubation of lactobacilli and pathogens, respectively, for 24h at 37ºC

Sample	MRS broth
L. rhamnosus A1	3.86
L. brevis A6	3.83
L. brevis B16	4.15
L. casei B5	4.65
L. plantarum B206	3.72
L. plantarum C0	4.38
L. rhamnosus C5	3.72
L. plantarum D4	3.96
L. plantarum E5	4.62
L. rhamnosus A1	4.74
Control*	6.24
	BHI broth
Escherichia coli ATCC 25922	6.73
Salmonella Typhimurium ATCC 14028	6.76
Shigella flexneri ATCC 25875	6.77
Control**	7.25

Legend: * MRS control broth, incubated without bacterial inoculation ****** BHI control broth, incubated without bacterial inoculation.

L. rhamnosus A1 and C5, *L. brevis* A6 and *L. plantarum* B206 showed the best results in co-culture antagonism, since they totally inhibited the growth of the three indicator pathogens, followed by *L. brevis* B16 which inhibited the growth of *S. flexneri* ATCC 25875 and *S.* Typhimurium ATCC 14028; *L. casei* B5, *L. plantarum* D4 and *L. plantarum* E5 which totally inhibited the growth of *S. flexneri* ATCC 25875 and *L. brevis* E35 which totally inhibited the growth of *S.* Typhimurium ATCC 1402.

CONCLUSIONS

Lactobacilli isolated from Minas artisanal cheeses produced in the Araxá region showed in vitro probiotic potential. From the tested samples, *L. brevis* A6 presented sensitivity to antimicrobial drugs, tolerance to artificial gastric juice and biliary salts and antagonism against reference pathogens. Considering the in vitro probiotic potential, *L. brevis* A6 was selected for future in vivo assays in order to fulfill its probiotic screening and will be used for the elaboration of probiotic fermented foods.

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