FULL ARTICLE

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Antagonistic activity of lactic acid bacteria isolated from Minas artisanal cheeses against *Brucella abortus*[*]

Atividade antagonista de bactérias lácticas isoladas de queijos Minas artesanais contra Brucella abortus

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ABSTRACT

This study aimed to evaluate methods for studying the *in vitro* antimicrobial activity of lactic acid bacteria (LAB) against *Brucella abortus* and to evaluate the antagonistic effect of LAB on the viability of this pathogen. A total of 18 LAB strains (*Lactobacillus plantarum,* n = 11; *Pediococcus acidilactici,* n = 1; *Lactobacillus rhamnosus,* n = 4; and *Lactobacillus brevis,* n = 2), isolated from Minas artisanal cheeses produced in three regions (Canastra, Campos das Vertentes, and Araxá) of Minas Gerais State, Brazil, were tested for their antimicrobial activity against *B. abortus* using three methods: spot-on-lawn, agar well diffusion assay, and antagonistic activity of the culture supernatants. None of the tested LAB strains could inhibit *B. abortus* in the spot-on-lawn and agar-well diffusion assays. The supernatants produced by LAB had an acidic pH, with intensity depending on bacterial growth and strain, and could inhibit the growth of *B. abortus*. In contrast, pH-neutralized (pH 7.0) LAB supernatants did not suppress the growth of *B. abortus.* The results showed that the best technique to study the *in vitro* antagonism of LAB against *B. abortus* was the antagonistic activity of culture supernatants. The growth of *B. abortus* may have been inhibited by acid production.

Keywords: Raw milk cheese. Milk microbiota. Antimicrobial activity. Brucellosis. Food security.

RESUMO

Este estudo teve como objetivo avaliar métodos de estudo *in vitro* da atividade antimicrobiana de bactérias lácticas contra *Brucella abortus* e avaliar o efeito antagônico das mesmas sobre a viabilidade deste patógeno. Um total de 18 amostras de bactérias lácteas (*Lactobacillus plantarum*, n = 11; *Pediococcus acidilactici*, n = 1; *Lactobacillus rhamnosus*, n = 4; e *Lactobacillus brevis*, n = 2), isoladas de exemplares de Queijo Minas Artesanal produzidos em três regiões (Canastra, Campos das Vertentes e Araxá) do estado de Minas Gerais, Brasil, foram testados quanto à sua atividade antimicrobiana contra *B. abortus* usando três métodos: spot-on-lawn, ensaio de difusão em poço e atividade antagonista de sobrenadante de cultura. Nenhuma das cepas testadas foi capaz de inibir *B. abortus* nos ensaios *spot-on-lawm* e de difusão em poço. Os sobrenadantes produzidos pelas bactérias lácteas apresentaram pH ácido, com intensidade dependente do crescimento bacteriano e da amostra, podendo inibir o crescimento de *B. abortus*. Em contraste, os sobrenadantes com pH neutralizado (pH 7,0) não inibiram o crescimento de *B. abortus*. Os resultados mostraram que a melhor técnica para estudar o antagonismo *in vitro* de bactérias lácteas contra *B. abortus* foi a atividade antagonista de sobrenadante de cultura. O crescimento de *B. abortus* pode ter sido inibido pela produção de ácido.

Palavras-chave: Queijo de leite cru. Microbiota do leite. Atividade antimicrobiana. Brucelose. Segurança alimentar.

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Introduction

Brucella abortus is a pathogenic agent responsible for significant economic losses to the cattle industry and public health (Corbel et al., 2006; Oliveira et al., 2017) and is mainly essential in developing countries due to failures in surveillance and disease control measures (Dadar et al., 2019; Godfroid et al., 2013). In Brazil, although the brucellosis control and eradication program has been implemented since 2001, the prevalence of the disease is heterogeneous, with high or low prevalence depending on the state (Ferreira Neto et al., 2016; Poester et al., 2002).

B. abortus has been isolated from raw milk, fresh cheese, and other dairy products available for retail sale in several countries, including Brazil, and has been involved in numerous cases of foodborne disease (Capuano et al., 2013; Hamdy & Amin, 2002; Kara & Akkaya, 2013; Wareth et al., 2014), especially in the commercialization of clandestine products, which may represent a risk to public health (Miyashiro et al., 2007; Paula et al., 2015).

Artisanal cheeses are produced with raw milk and represent a significant proportion of dairy products, contributing to the preservation of the socio-cultural identity, and are economically significant in several regions of the world, especially in Latin America (Menezes, 2011). The Minas artisanal cheese is a traditional dairy product in Brazil and has a rich microbiota derived from raw milk and an endogenous starter culture used in cheesemaking (Martins et al., 2015; Oliveira et al., 2018). The presence of lactic acid bacteria (LAB) in this type of cheese allows the development of its typical sensory characteristics (Castro et al., 2016; Luiz et al., 2016; Resende et al., 2011) and is essential for its safety since they compete for nutrients with other microorganisms and can synthesize many antimicrobial compounds, including organic acids, hydrogen peroxide, diacetyl, and bacteriocins, which can inhibit the growth of pathogenic bacteria (Quigley et al., 2013; Ross et al., 2002).

The effectiveness of LAB in the preservation of milk and dairy products through its antagonistic activity has been consistently demonstrated for some foodborne pathogens of public health importance, such as *Escherichia coli, Salmonella* spp., *Staphylococcus aureus,* and *Listeria monocytogenes* (Andrade et al., 2014; Dal Bello et al., 2012; Menéndez et al., 2001). This effectiveness is due to different inhibitory activities, such as bacteriocins and acid production; however, few studies have focused on zoonotic agents, such as *Brucella* spp. Although some studies have shown that LAB can reduce the pH of milk and dairy products, which could influence the survival time of *Brucella* spp. in these products (Davies & Casey, 1973; el-Daher et al., 1990; Kuzdas & Morse, 1954), no studies have demonstrated the antagonistic activity of LAB against bacteria from the genus *Brucella*.

Therefore, this study aimed to evaluate different methods for studying the *in vitro* antimicrobial activity of LAB against *B. abortus* and to evaluate the antagonistic effect of LAB isolated from cheese on the viability of this pathogen.

Materials and Methods

Bacterial strains and culture conditions

A total of 18 LAB (Table 1) and three *B. abortus* strains were used in this study. LAB strains were isolated from Minas artisanal cheeses produced in three regions (Canastra, Campos das Vertentes, and Araxá) located in Minas Gerais, Brazil (Castro et al., 2016; Luiz et al., 2016; Resende et al., 2011; Sales, 2015; Sant'anna et al., 2017), and the strains were selected based on their inhibitory potential against some bacterial pathogens, as assessed by previous studies using spot-on-lawn antagonism tests (Andrade et al., 2014; Costa et al., 2013; Sant'anna et al., 2017; Silva et al., 2019). *B. abortus* reference strains 2308 and 544 (ATCC 23448) (Miranda et al., 2015; Poester et al., 2006) and *B. abortus* field strain 2683 (biovar 3, isolated from milk) (Dorneles et al., 2014) were used as an indicator of bacteria in antimicrobial activity tests.

LAB strains were grown in Man–Rogosa–Sharpe (MRS) broth (BD, USA) at 37°C for 24 h under aerobic conditions, while *B. abortus* strains were grown on Tryptose agar (BD, USA) at 37°C for 24 h in 5% CO_2. Then, 10^8 colony-forming units (CFU)/mL and 10^4 CFU/mL of LAB and *B. abortus* strains were prepared in phosphate-buffered saline (PBS

Table 1 – Lactic acid bacteria isolated from Minas artisanal cheeses produced in the regions of Serra da Canastra, Araxá, and Campo das Vertentes used in *in vitro* antimicrobial activity assays against *Brucella abortus*

Lactic acid bacteria strain	Species	References
Campos das Vertentes		Sant'anna et al. (2017)
LP1	*Lactobacillus plantarum*	
LP2	*Lactobacillus plantarum*	
LP3	*Lactobacillus plantarum*	
LP4	*Lactobacillus plantarum*	
LP7	*Lactobacillus plantarum*	
PA2	*Pediococcus acidilactici*	
Araxá		Luiz et al. (2016), Sales (2015)
A1	*Lactobacillus rhamnosus*	
A6	*Lactobacillus brevis*	
B16	*Lactobacillus brevis*	
B206	*Lactobacillus plantarum*	
C5	*Lactobacillus rhamnosus*	
E5	*Lactobacillus plantarum*	
Serra da Canastra		Resende et al. (2011)
A8	*Lactobacillus rhamnosus*	
B17	*Lactobacillus plantarum*	
B19	*Lactobacillus plantarum*	
B4	*Lactobacillus rhamnosus*	
B7	*Lactobacillus plantarum*	
D8	*Lactobacillus plantarum*	

0.01 M, pH 7.2) and used in the following tests. Each strain's CFU number was confirmed retrospectively using the drop counting method (Miles et al., 1938). Briefly, after the bacterial suspension reached an optical density (OD) of 1.00 (OD_{600}) for LAB strains and 0.10 (OD_{600}) for *B. abortus*, 20 µL of 10^{-1} to 10^{-5} culture dilutions were inoculated equidistant on MRS and Tryptose agar, respectively, followed by incubation at 37ºC for 24 h for LAB strains and 72 h for *B. abortus* strains, when colony counting was performed.

Growth test of Brucella abortus in different culture media

To evaluate the ability of *B. abortus* to grow in different media that could be used in antagonism tests, *B. abortus* strains (100 µL of 10^4 CFU suspension) were inoculated in 3.5 mL of semisolid Tryptose agar (0.75% agar) at 50°C and spread (using Drigalski spatula) on plates containing the following media: brain heart infusion (BHI) agar (Oxoid, England), BHI agar supplemented with D-glucose (Synth, São Paulo, Brazil) (final concentration of 20 g/L), MRS agar (BD), M17 agar (BD), and Mueller Hinton (MH) agar (BD). All plates were incubated at 37°C for 72 h in 5% CO_2.

The growth of *B. abortus* was also tested in Tryptose broth (BD) plus MRS broth in different proportions (v/v), 1:2, 1:3, and 1:5, and the medium was inoculated with the standardized inoculum of *B. abortus* strains (100 µL of 10^4 CFU suspension).

Antagonistic activity assays of LAB against B. abortus

Three different assays were tested to determine the *in vitro* antagonistic activity of LAB strains against *B. abortus*: spot-on-lawn antagonism assay, agar-well diffusion assay, and antagonistic activity of the culture supernatants of the LAB assay.

E. coli ATCC 25922 (Andrade et al., 2014; Oliveira et al., 2018; Sant'anna et al., 2017; Silva et al., 2019) was used as a susceptible control. It was grown on MacConkey agar (BD) at 37°C for 24 h. The colonies were resuspended in PBS until an OD of 0.08–0.13, corresponding to 10^8 UFC/mL. Next, the bacterial suspension was serially diluted in PBS, and 100 µL of 10^4 CFU suspension was used in all assays.

All experiments were performed in triplicate. The results for all assays were considered qualitative, expressed by the presence or absence of inhibition.

Spot-on-lawn antagonism assay

The antagonism of the LAB strains against *B. abortus* was examined using the spot-on-lawn assay (Tagg et al., 1976). Briefly, 10 µL of each LAB was spotted in the center of Petri dishes containing MRS agar, BHI agar, or BHI agar plus D-glucose and incubated aerobically at 37°C for 24 h. The bacteria grown in the spots were then killed by exposure to 1.0 mL of chloroform for 30 min under a UV lamp. The residual chloroform was allowed to evaporate for 30 min. After inactivation, the plates were incubated at 37°C for 12 h.

Subsequently, 3.5 mL of semisolid Tryptose agar (0.75% agar) maintained at 50°C was inoculated with 100 µL of 10^4 CFU/mL suspension of *B. abortus* strains or *E. coli* ATCC 25922 and spread over plates, covering the LAB spots. The plates were then incubated at 37°C for 72 h in 5% CO_2 for *B. abortus* or at 37°C for 24 h aerobically for *E. coli* before checking for the presence of the inhibition halo.

Agar-well diffusion assay

The agar-well diffusion assay was performed as previously described (Yang et al., 2012). An inoculum of 100 µL of a 10^4 CFU/mL suspension of each LAB strain was inoculated in 10 mL MRS broth and incubated at 37°C for 24 h. The drop-counting method described previously verified the number of CFUs in the bacterial suspension. After incubation, LAB cultures were centrifuged at 14,000 × g for 5 min at room

temperature. The supernatants were filtered through a 0.22 µm filter (Kasvi, Brazil), and pH was measured using a pH meter PHS-3B (Labmeter, China). One milliliter of each *B. abortus* strain or *E. coli* ATCC 25922 containing 10^3 CFU/mL was inoculated into 15 mL of semisolid (0.75% agar) Tryptose or BHI agar, respectively, maintained at 50°C and then spread on a Petri dish (90 × 15 mm). After solidification for 30 min, three 5-mm diameter wells were cut on agar, and 35 µL of cell-free supernatant from each LAB was added to each well. Plates were incubated at 37°C for 72 h in 5% CO_2 for *B. abortus* or at 37°C for 24 h under aerobic conditions for *E. coli* ATCC 25922; the presence or absence of the inhibition halo was then evaluated.

Antagonistic activity of the culture supernatants of the LAB assay

The antagonistic activity of LAB culture supernatants against *B. abortus* was performed as previously described (Hütt et al., 2006) with modifications. Briefly, 200 µL of 10^4 CFU/mL suspensions of each LAB were inoculated in 30 mL MRS broth and incubated at 37°C for 24 h. After incubation, the cultures were centrifuged at 2,000 × g for 10 min at room temperature, and the pH was measured using a pH meter. The volume of the supernatant was divided into two parts of 15 mL, and one was neutralized to pH 7.0 ± 0.1 with NaOH (1 M). Both pH-adjusted and pH-unadjusted supernatants were filtered through a 0.22 µm membrane. Bacterial counts in culture were performed retrospectively using the drop counting method as previously described.

A volume of 2.0 mL of each cell-free supernatant from LAB cultures was mixed with equal volumes of Tryptose broth. Sterility was confirmed by spreading the mixture on MRS plates and incubating at 37°C for 12 h. After this period, the mixture was inoculated with 10^3 CFU of *B. abortus* strains and cultured for 24 h at 37°C in 5% CO_2. Then, 100 µL of these cultures were spread on Tryptose agar plates and incubated at 37°C for 72 h in 5% CO_2 to verify the occurrence of inhibition, partial inhibition, or no inhibition of *B. abortus* growth.

As a control, *E. coli* ATCC 25922 inoculated into BHI broth was mixed and incubated similarly to LAB supernatants. MacConkey agar plates were inoculated with 100 µL of previous cultures and incubated at 37°C under aerobic conditions for 24 h to evaluate the inhibition of the growth of *E. coli*.

Results

Growth of Brucella abortus in culture media

B. abortus strains 544, 2308, and 2683 could not grow on MRS agar, M17, or semisolid Tryptose agar when this medium was spread on plates containing MRS agar. However, all *B. abortus* strains tested were able to grow on BHI, BHI medium containing 20 g/L of D-glucose, equivalent to the D-glucose concentration of MRS and MH agar.

B. abortus strains could not grow in MRS broth; however, we observed that the mixture of Tryptose broth and MRS broth, in all proportions tested, allowed the growth of *B. abortus* strains. Therefore, a 1:2 ratio was used to test the antagonistic activity of LAB supernatants.

Antagonistic activity assays of LAB against B. abortus

None of the tested LAB strains could inhibit *B. abortus* strains in the spot-on-lawn antagonism assay using BHI or BHI medium plus D-glucose for the growth of the indicator microorganism. *E. coli* ATCC 25922 was antagonized by LAB strains only when MRS medium was used.

The results of the agar-well diffusion assay are presented in Table 2. LAB counts in MRS broth for supernatant production ranged from 10^7 to 10^9 CFU/mL and exhibited an acid pH of 3.89 to 5.96 (Table 2). Despite the low pH and high concentration of LAB in the supernatant, none of the tested *B. abortus* strains were inhibited in this assay. Furthermore, no inhibition halos were observed when *E. coli* ATCC 25922 was used as an indicator bacterium.

The results of the antagonistic activity of the culture supernatants of LAB strains against *B. abortus* are shown in Figure 1. The supernatants produced by LAB exhibited acidic pH, ranging from 3.85 to 6.1, depending on the LAB strain and bacterial concentration, which ranged from 10^5 to 10^9 CFU/mL (Table 2). Of the 18 strains tested, 13 LABs (72.22%), with faster growth and higher concentrations in the MRS broth, reduced the pH of the medium below 4.65 and were able to inhibit all B. abortus strains tested thoroughly. When pH was between 4.65 and 5.0, LABs could partially inhibit *B. abortus* growth, and at pH >5.6, no inhibition was observed (Figure 1). In contrast, pH 7.0 neutralized LAB supernatants did not inhibit the growth of the tested *B. abortus* or *E. coli* strains (Figure 1).

Discussion

Several protocols can be used for antagonism studies, but the MRS medium, which is selective for cultivating LAB, is traditionally used (Costa et al., 2013; Grimoud et al., 2010; Tulini et al., 2013). However, the inability of *B. abortus* to grow in MRS makes it impossible to use antagonism techniques based on MRS to evaluate the antimicrobial activity of LAB against this pathogen.

Table 2 – Count (CFU/mL) and supernatant pH of cultures of lactic acid bacteria isolated from Minas artisanal cheeses used in the agar-well diffusion assay and antagonistic activity of the culture supernatant assay against *Brucella abortus* 544, 2308, and 2683, and *Escherichia coli* ATCC 25922

LAB strain	Agar-well diffusion assay		Antagonistic activity of the culture supernatants assay	
	LAB count in the supernatant (CFU/mL)	Supernatant pH[a]	LAB count in the supernatant (CFU/mL)	Supernatant pH[a]
Campos das Vertentes				
LP1	5.55×10^8	3.89 ± 0.07	1.49×10^9	3.85 ± 0.07
LP2	7.25×10^8	3.89 ± 0.03	1.34×10^9	3.88 ± 0.04
LP3	6.65×10^8	3.90 ± 0.09	1.01×10^9	3.88 ± 0.07
LP4	1.00×10^9	3.90 ± 0	2.20×10^9	3.85 ± 0.03
LP7	6.40×10^8	3.97 ± 0	1.73×10^9	3.93 ± 0.06
PA2	4.50×10^8	3.92 ± 0.01	1.88×10^9	3.86 ± 0.02
Araxá				
A1	3.80×10^8	4.41 ± 0.06	9.35×10^8	4.21 ± 0.09
A6	6.35×10^8	4.43 ± 0.03	1.83×10^9	4.21 ± 0.03
B16	5.40×10^8	5.15 ± 1.5	3.45×10^5	6.10 ± 0.21
B206	2.85×10^8	4.35 ± 0.00	9.88×10^8	4.21 ± 0.04
C5	3.40×10^8	4.10 ± 0.03	1.70×10^9	4.11 ± 0.03
E5	4.55×10^8	5.32 ± 0.39	1.42×10^9	4.30 ± 0.19
Serra da Canastra				
A8	3.71×10^7	5.80 ± 0.41	1.21×10^8	5.87 ± 0.17
B17	7.10×10^8	3.95 ± 0.03	3.08×10^9	3.89 ± 0.07
B19	9.30×10^8	4.23 ± 0.29	1.13×10^9	4.14 ± 0.16
B4	3.65×10^8	4.90 ± 0.13	2.73×10^9	5.00 ± 0.29
B7	2.30×10^7	5.96 ± 0.26	2.35×10^8	5.69 ± 0.13
D8	5.40×10^8	4.52 ± 0.32	2.30×10^9	4.65 ± 0.21

[a]Potential of hydrogen.

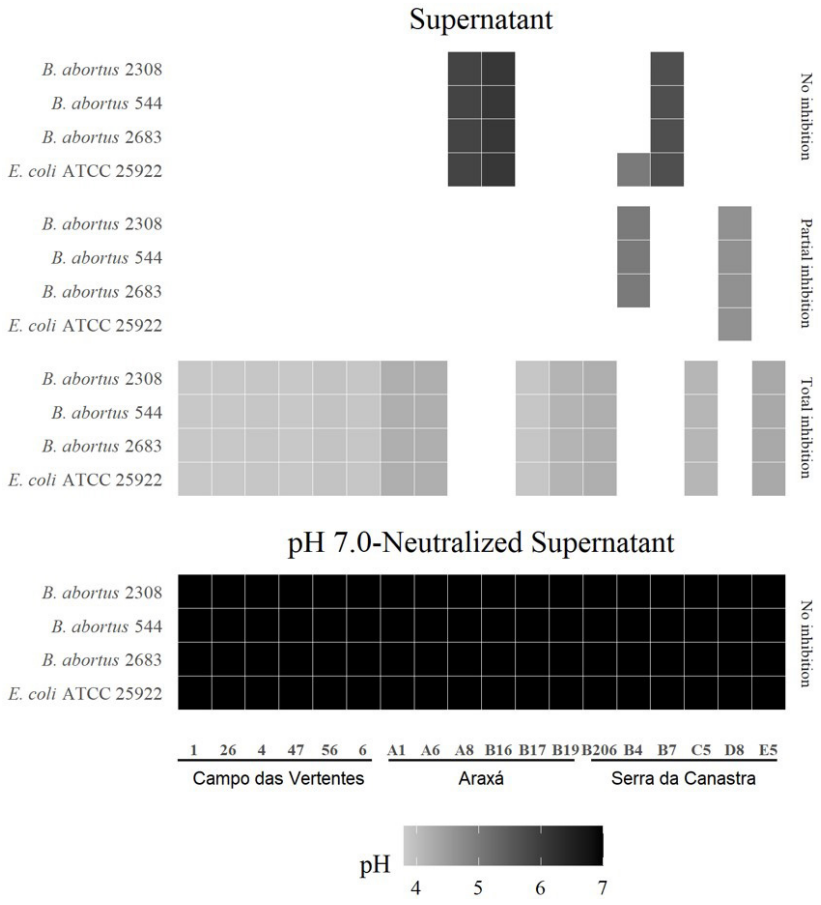

Figure 1 – Antagonistic effect of supernatant and pH 7.0 neutralized the supernatant of cultures of lactic acid bacteria isolated from Minas artisanal cheeses against *Brucella abortus* 544, 2308, and 2683, and *Escherichia coli* ATCC 25922.

The use of BHI media was also not promising in the spot-on-lawn assay, as no inhibition halo could be observed in *B. abortus* or even in *E. coli* ATCC 25922. However, *E. coli* ATCC 25922 was already observed to be inhibited by the tested LAB strains in this and previous studies when MRS was used in the assay (Andrade et al., 2014; Costa et al., 2013; Oliveira et al., 2018; Sant'Anna et al., 2017; Silva et al., 2019). These results could be due to the lower concentration of D-glucose in the BHI medium compared to the MRS, as the fermentation of glucose into lactic acid, lactate, carbon dioxide, and ethanol is essential for the antagonistic activity of LAB against pathogens (Ross et al., 2002). Supplementation with sugar in this medium could promote greater acid production by LAB. Therefore, we supplemented BHI at a final concentration of 20 g/L of D-glucose, but even with this supplementation, no inhibition of *B. abortus* or *E. coli* ATCC 25922 growth was observed. However, BHI was used to test the antagonism of *L. monocytogenes* by LAB (Harris et al., 1989) and is also used as an alternative medium for the detection of LAB cultures with antimicrobial potential, producing mainly substances of proteinaceous nature, such as bacteriocins (Moraes et al., 2010), due to its low glucose concentration (2%), which minimizes acid production. Culture medium plays an essential role in *vitro* antagonism assays and should provide an ideal nutritional environment to support the growth of all microorganisms used in the test (Huys et al., 2002). The results of the spot-on-lawn assay using *E. coli* ATCC 25922 on MRS, BHI, or BHI agar supplemented with D-glucose showed that the composition of the culture medium influences the antagonistic activity of LAB. Although LAB can grow in all media, the production and diffusion of antagonistic substances did not occur similarly in all culture media (Djadouni & Kihal, 2012; Zamfir et al., 2016). Similar to our findings, other studies also demonstrated that the antagonistic activity of LAB against pathogens with the production of substances, such as bacteriocins and hydrogen peroxide, commonly occurs in tests performed with MRS and was not observed in the same way in BHI (Mataragas et al., 2004; Moraes et al., 2010; Zalán et al., 2005). MRS contains nutrients such as amino acids, B-complex vitamins, purines, and pyrimidines bases, among others, absent in BHI, which may be required to produce substances with antagonistic effects on pathogens (Djadouni & Kihal, 2012; De Kwaadsteniet et al., 2005).

The results of the agar-well diffusion assay demonstrated that despite the low pH of the supernatant produced by the tested LAB strains (Table 2), there was no inhibition of *E. coli* ATCC 25922 or *B. abortus* strains. Antagonism assay by agar-well diffusion may exhibit varying degrees of inhibitory activity against different pathogens, probably due to the LAB strains used, acidic pH, presence and concentration of hydrogen peroxide, or bacteriocin in the supernatant (Bogovič-Matijašić & Rogelj, 1998). In addition, in this technique, the amount of antagonistic substance is limited, and the pathogen continues to grow because the condition of the medium promotes its development (Harris et al., 1989). Therefore, based on our results, the agar-well diffusion assay was unsuitable for studying *B. abortus* antagonism by LAB.

Indeed, the antagonistic activity of the culture supernatants of the LAB assay was the only assay capable of testing the antagonism of *B. abortus*, probably by providing an ideal nutritional environment to support the growth of all microorganisms used in the test, LAB, and *B. abortus* (Huys et al., 2002). The three virulent *B. abortus* strains tested were inhibited by most of the tested LAB strains isolated from Minas artisanal cheese (13/18 strains, 72.22%) (Figure 1). Moreover, two LAB strains isolated from Serra da Canastra partially inhibited the growth of the *B. abortus* strains. Only three out of the 18 tested LAB strains, one from Araxá and two from the Serra da Canastra region, did not inhibit the growth of the tested *B. abortus*. These results showed that antagonistic substances were present in the supernatant produced by the LAB strains tested. Moreover, when the LAB culture supernatants were neutralized to pH 7.0, their inhibitory effect on the tested *B. abortus* strains was reversed (Table 2), demonstrating that pH is a factor that directly or synergistically influences the inhibition of *B. abortus* (Figure 1). An assay conducted with a culture medium containing hydrochloric acid showed that pH 5 allowed the survival of *B. melitensis* for a period of <3 weeks, while at pH <4, the bacteria survived for <24 h (el-Daher et al., 1990), corroborating our results. In contrast, *B. abortus* (10^6–10^7 CFU/mL) has been shown can survive for 8 days at pH 4 and a temperature of 5°C in citrate/phosphate buffer (0.1 M) (Davies & Casey, 1973).

Although the literature reports different survival periods for *Brucella* spp. in culture medium with low pH, in studies evaluating the survival of *Brucella* spp. in cheese, yogurt, and buttermilk, a decrease in pH during fermentation was an essential factor in reducing the viability and survival of this pathogen (el-Daher et al., 1990; Méndez-González et al., 2011; Zúñiga Estrada et al., 2005). In addition to pH, the organic acids in the LAB supernatants also diffuse through the bacterial membrane, releasing H^+ ions, causing perturbations of intracellular processes, alterations in membrane permeability, and bacterial death (Ivey et al.,

2013). However, we did not find studies that evaluated the *in vitro* survival of *Brucella* spp. in lactic acid, the leading organic acid produced by LAB, which is responsible for the decrease in the pH of dairy products (Sousa et al., 2001). The presence of bacteriocins in the supernatant produced by LAB has also been inferred to be an essential inhibition mechanism along with acidic pH. This was observed for *Mycobacterium bovis*, another zoonotic pathogen tested against some of the LABs isolated from Minas artisanal cheese used in our study (Oliveira et al., 2018). However, in contrast to that study, our results did not show inhibition of *B. abortus* when pH was neutralized, indicating the absence of bacteriocins active against *B. abortus* in the supernatant of the tested LAB strains or that their effects were masked by environmental acidification. Our study demonstrated that the organic acids produced by fermentative metabolism in LAB were the essential substances responsible for inhibiting *B. abortus*.

It should be noted that in this study, we evaluated the effect of each LAB individually on the survival of *B. abortus*. Our *in vitro* results showed the potential use of these LAB strains to increase the safety of Minas artisanal cheese. However, in cheeses, there are a variety of LAB strains that act synergistically, producing bacteriocins in addition to organic acids and other antagonist substances, which, together with changes in intrinsic and extrinsic factors of cheese, can influence the survival of pathogens and cause total inhibition of *Brucella* spp. in cheeses (Beresford & Williams, 2004; Gálvez et al., 2010). Another study conducted by our research group showed that the reduction in pH and count of microorganisms, including LAB, in cheeses made with endogenous starter culture obtained from the Campos das Vertentes region, along with other parameters, was correlated with inhibition of *B. abortus* during the ripening process (Mussi, 2014). Therefore, the results of *in situ* assays using starter cultures from the same region as some of the LAB strains used in this study corroborate our *in vitro* findings.

Conclusions

Among the *in vitro* antagonism assays evaluated in this study, the antagonistic activity of the culture supernatant was the only one that produced beneficial results against the tested *B. abortus* strains, demonstrating the potential inhibition of *B. abortus* by LAB strains due to organic acid production and consequent pH reduction.

Conflict of Interest

None of the authors have financial or personal relationships that could inappropriately influence or bias the paper's content.

Ethics Statement

The authors declare no competing interests.

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