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Microbiological Quality and Safety of Raw Milk and Soft Cheese and Detection of Autochthonous Lactic Acid Bacteria with Antagonistic Activity Against *Listeria monocytogenes*, *Salmonella* Spp., and *Staphylococcus aureus*

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Abstract

This study aimed to characterize the microbiological quality and safety of raw milk and soft cheese, verifying possible associations between microbial populations, and the detection of lactic acid bacteria (LAB) with antagonistic activity against foodborne pathogens. Raw milk ($n = 36$) and soft cheese ($n = 18$) samples were collected and submitted for the analysis of mesophilic aerobes, total coliforms, *Escherichia coli*, LAB, coagulase-positive *Staphylococcus* (CPS), *Listeria monocytogenes*, and *Salmonella* spp. In all, 389 LAB isolates were randomly selected and submitted for antagonistic tests against *L. monocytogenes*, *St. aureus*, *Salmonella* Typhimurium, and *Lactobacillus sakei*. The samples presented high counts of mesophilic aerobes, total coliforms, and LAB, and also high and significant correlation indices between these populations. Low levels of CPS and *E. coli* were observed, as well as an absence of *Salmonella* spp. and *L. monocytogenes*. A substantial portion of the analyzed samples presented LAB cultures with antagonistic activity, but not against *Salmonella* Typhimurium. The obtained results indicate the antimicrobial potential of the autochthonous microbiota of raw milk and soft cheese. Despite the spoilage potential, the LAB present in the studied food products can be isolated and properly characterized as antagonistic cultures, to be used in bioconservation studies for pathogen control in foods.

Introduction

Milk produced in Brazil is usually obtained from dairy farms characterized by low-quality technology and poor hygienic conditions, resulting in a final product with poorly microbiological quality with high counts of indicator microorganisms (Pereira *et al.*, 1999; Loguercio and Aleixo, 2001; Carmo *et al.*, 2002; Feitosa *et al.*, 2003). It is estimated that 40% of the Brazilian milk is produced without any official inspection (Nero *et al.*, 2008), being marketed as fluid milk or dairy products, especially soft cheeses, without proper heat treatment and posing as potential hazards for consumers.

These products have been previously associated with several food poisoning cases and outbreaks caused by *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, and verotoxigen *Escherichia coli* (de Buyser *et al.*, 2001; Oliver *et al.*, 2005; Vaillant *et al.*, 2005). A source of these foodborne pathogens is the raw milk itself, but inappropriate handling, manufacturing, and storage are also considered significant sources of contamination.

These cases and outbreaks are mainly reported in developed countries, where they are properly reported and characterized by official fiscalization organs. Also, these countries are usually characterized by highly controlled hygienic conditions of milk producing, suggesting good microbiological quality of raw milk and dairy products (Gaya *et al.*, 1998; de Buyser *et al.*, 2001; Guerra *et al.*, 2001; Leclerc *et al.*, 2002). However, the occurrence of foodborne pathogens tends to decrease as the microbiological quality of food products is poor, conditions typically associated with raw milk and raw milk products produced in Brazil (Jay, 1995; Dhanashree *et al.*, 2003; Nero *et al.*, 2008). This paradox suggests that the autochthonous microbiota of these products, when present at high levels and depending on the predominant groups, interfere with the development of foodborne pathogens (Dodd *et al.*, 2007; Nero *et al.*, 2008).

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The main promoters of these conditions in foods are lactic acid bacteria (LAB). LAB are part of the autochthonous microbiota of milk and dairy products (Franciosi *et al.*, 2009) and are able to produce several substances with antimicrobial activity, such as organic acids, hydrogen peroxide, dyacetil, CO_2, and bacteriocins (Deegan *et al.*, 2006). During their development, LAB are able to produce these substances and inhibit foodborne pathogens that are eventually present in the food. This interference can occur in the food itself or during the enrichment steps of conventional methodologies of pathogen isolation.

The aim of this study was to characterize the microbiological quality and safety of raw milk and soft cheese, verifying possible associations between their distinct microbiological populations, as well as to detect naturally occurring LAB of these food products with antagonistic activity against foodborne pathogens.

Materials and Methods

Samples and dilution

Raw milk samples ($n = 36$), obtained direct from dairy farm bulk tanks, and raw milk soft cheese samples ($n = 18$), obtained from dairy farms and markets, were collected in aseptic conditions in the region of Viçosa, Minas Gerais, Brazil, and kept under refrigeration at 4°C until analysis, when they were thoroughly homogenized and submitted to 10-fold dilution using NaCl 0.85%.

Microbiological characterization

The collected samples were submitted for microbiological analysis for enumeration of hygiene indicator microorganisms (mesophilic aerobes [MA], total coliforms [TC], and *E. coli* [EC]), LAB, and coagulase-positive *Staphylococcus* (CPS), and detection of *L. monocytogenes* and *Salmonella* spp. MA, TC, and EC were enumerated using Petrifilm™ AC and EC plates (3M Microbiology, St. Paul, MN) incubated at 35°C for 48 h. LAB populations were estimated using de Mann–Rogosa–Sharpe (MRS) agar (Oxoid, Basingstoke, Hampshire, United Kingdom) incubated at 35°C for 48 h under anaerobiosis (Anaerobac, Probac do Brasil, São Paulo, SP, Brazil) (Wehr and Frank, 2004). CPS were enumerated using Baird–Parker agar (Oxoid) incubated at 35°C for 48 h, followed by catalase, coagulase, and thermonuclease tests of typical and atypical colonies (Wehr and Frank, 2004). All results were expressed as colony-forming units per milliliter or grams (CFU/mL or g). *L. monocytogenes* and *Salmonella* spp. were studied by the conventional procedures described by Wehr and Frank (2004), and the obtained results were expressed as presence or absence of the pathogens in 25 mL or g of the sample.

Reference strains

For antagonistic tests, *L. monocytogenes* ATCC 7644, *Salmonella* Typhimurium ATCC 41028, *St. aureus* ATCC 14458, *Lactobacillus sakei* ATCC 15521, and *Lb. sakei* 2a (16) were kept under refrigeration in trypticase soya agar (Oxoid) or MRS agar (Oxoid) slants, and in the moment of use, they were recovered in trypticase soya broth (Oxoid) or MRS broth (35°C for 24 h). The obtained cultures were diluted in trypticase soya broth or MRS broth to achieve turbidity similar to scale 1 of McFarland (3×10^8 CFU/mL).

Antagonistic activity of naturally occurring LAB

Three-hundred and eighty-nine colonies were randomly selected from MRS agar plates for antagonistic test. First, all cultures were streaked on MRS agar plates (35°C for 24–48 h) and isolated colonies were transferred to MRS broth (Oxoid) (35°C for 24 h). The obtained cultures were diluted in MRS broth to achieve turbidity similar to scale 1 of McFarland. To identify the antagonistic activity, 2 μL of each selected LAB culture was spotted onto the surface of four distinct plates containing M17 agar supplemented by lactose (10%) (Oxoid), followed by incubation at 35°C for 24 h. Then each plate was overlayed with 8 mL of brain–heart infusion with 0.75% agar (Oxoid) containing approximately 10^5 CFU/mL of a target strain: *L. monocytogenes* ATCC 7644, *Salmonella* Typhimurium ATCC 14028, *St. aureus* ATCC 14458, or *Lb. sakei* ATCC 15521 (previously known to be sensitive to LAB antagonistic substances). The plates were incubated at 35°C for 24 h and examined for the for-

Table 1. Frequencies of Raw Milk and Soft Cheese with Distinct Levels of Mesophilic Aerobes, Total Coliforms, *Escherichia coli*, Lactic Acid Bacteria, and Coagulase-Positive *Staphylococcus*

Samples	*Counts (CFU/mL or g)*	*MA*, n *(%)*	*TC*, n *(%)*	*EC*, n *(%)*	*LAB*, n *(%)*	*CPS*, n *(%)*
Raw milk	Until 10^2	1 (2.8)	17 (42.7)	34 (94.4)	0	19 (52.8)
	10^2 to 10^3	0	8 (22.2)	2 (5.6)	2 (5.6)	5 (13.9)
	10^3 to 10^4	4 (11.1)	4 (11.1)	0	16 (44.4)	4 (11.1)
	10^4 to 10^5	13 (36.1)	3 (8.3)	0	7 (19.4)	6 (16.7)
	10^5 to 10^6	10 (27.8)	4 (11.1)	0	8 (22.2)	2 (5.6)
	Higher than 10^6	8 (22.2)	0	0	3 (8.3)	0
Total		36 (100)	36 (100)	36 (100)	36 (100)	36 (100)
Soft cheese	Until 10^4	0	3 (16.7)	8 (44.4)	3 (16.7)	17 (94.4)
	10^4 to 10^5	0	1 (5.6)	7 (38.9)	0	1 (5.6)
	10^5 to 10^6	0	6 (33.3)	3 (16.7)	2 (11.1)	0
	10^6 to 10^7	2 (11.1)	6 (33.3)	0	6 (33.3)	0
	10^7 to 10^8	9 (50.0)	2 (11.1)	0	7 (38.9)	0
	Higher than 10^8	7 (38.9)	0	0	0	0
Total		18 (100)	18 (100)	18 (100)	18 (100)	18 (100)

MA, mesophilic aerobes; TC, total coliforms; EC, *Escherichia coli*; LAB, lactic acid bacteria; CPS, coagulase-positive *Staphylococcus*.

mation of an inhibition halo around the spotted LAB, indicating unspecific antagonistic activity of the tested culture. In all plates, a culture of *Lb. sakei* 2a was also spotted as positive control of antagonistic LAB, once it is described as a bacteriocinogenic strain with wide-activity spectrum (de Martinis and Franco, 1998).

Data analysis

The obtained results were categorized considering the counts of hygiene indicator microorganisms and LAB, and the presence or absence of the studied foodborne pathogens. The obtained counts were converted to $\log_{10}$ and LAB values were compared to MA, TC, EC, and CPS by linear regression ($p < 0.05$) to verify possible correlations between these microbiological groups. Finally, the frequencies of the samples that presented LAB cultures with antagonistic activity were calculated, considering the indicator microorganism. The same analysis was conducted for the tested LAB cultures. All analyses were conducted using the software Statistica 7.0 (StatSoft, Tulsa, OK).

Results

The frequencies of raw milk and soft cheese samples categorized according to the levels of contamination by indicator microorganisms are shown in Table 1. High counts of MA, coliforms, and LAB, contrasting with the low levels of EC and CPS, and absence of *L. monocytogenes* and *Salmonella* spp can be observed.

Correlation parameters between microbial populations in the analyzed samples are presented in Table 2, where high and significant values between LAB and hygiene indicator microorganisms can be observed.

Antagonistic activity was evaluated considering the production of unspecific antimicrobial substances. Considering the obtained results, a high frequency of samples containing antagonistic LAB was observed, mainly with the activity against *L. monocytogenes* and *Lb. sakei* (Table 3). Considering the tested LAB cultures, it was possible to verify antimicrobial activity against more than one target pathogen simultaneously, also against *L. monocytogenes* and *Lb. sakei* (Table 4). None of the tested LAB presented antagonistic activity against *Salmonella* Typhimurium.

Discussion

MA are often considered as an important microbiological parameter for milk and dairy products quality, and when present at high levels (higher than 10^5 CFU/mL) indicate serious deficiencies in production hygiene, whereas values lower than 20,000 CFU/mL reflect good sanitary practices (Chambers, 2002). Coliforms are also used as microbiological parameters to validate the quality of milk and dairy products, and values higher than 100 CFU/mL are considered proof of unsatisfactory production practices leading to environmental contamination (Chambers, 2002). Considering these values, a high frequency of raw milk and cheese samples produced in poor microbiological conditions can be observed (Table 1), suggesting the presence of pathogenic microorganisms (ICMSF, 1988). Similar results were obtained by studies on microbiological quality and safety of milk and dairy products produced in distinct Brazilian regions and other countries, with equivalent dairy production conditions (Soler *et al.*, 1995; Chye *et al.*, 2004; Arcuri *et al.*, 2006; Nero *et al.*, 2008).

Table 2. Statistical Parameters of Correlation Between Lactic Acid Bacteria and Mesophilic Aerobes, Total Coliforms, *Escherichia coli*, and Coagulase-Positive *Staphylococcus* Counts Obtained from Raw Milk and Soft Cheese Samples

Comparison (x:y)	n	r	r^2	p	a	b	*mv*
All samples							
LAB:MA	49	0.93	0.87	0.00	1.12	−1.46	0.52
LAB:TC	41	0.88	0.78	0.00	0.80	2.42	1.90
LAB:EC	9	0.88	0.77	0.00	1.09	2.13	2.98
LAB:CPS	19	0.40	0.16	0.09	0.62	2.29	1.39
Raw milk							
LAB:MA	31	0.80	0.64	0.00	0.84	−0.07	0.53
LAB:TC	27	0.38	0.14	0.05	0.31	3.39	2.04
LAB:EC	7	0.24	0.06	0.60	0.35	3.16	2.61
LAB:CPS	16	0.57	0.32	0.02	0.47	2.31	0.47
Soft cheese							
LAB:MA	18	0.78	0.61	0.00	1.41	−3.65	0.50
LAB:TC	14	0.62	0.39	0.02	0.51	4.50	1.64
LAB:EC	–	–	–	–	–	–	–
LAB:CPS	3	0.97	0.95	0.15	1.52	1.56	6.33

p-Values higher than 0.05 indicate not significant correlation indexes.

n, number of repetitions; *r*, correlation; r^2, coefficient of determination; *p*, level of significance; *a*, slope; *b*, intercept; mv, mean variance.

Despite the high counts of MA and TC, the analyzed samples showed low levels of EC and SCP (Table 1). In addition, none of the evaluated samples presented *Salmonella* spp. or *L. monocytogenes*. Similar results were obtained previously by Akineden *et al.* (2008), Kongo *et al.* (2008), Nero *et al.* (2008), Dhanashree *et al.* (2003), and Cordano and Rocourt (2001), who observed a low pathogen incidence associated with poor microbiological quality in animal-based products. In contrast, when better microbiological quality was observed, selected studies show an increase in pathogen detection (Guerra *et al.*, 2001; Rudolf and Scherer, 2001; Leclerc *et al.*, 2002; D'Amico *et al.*, 2008; Ghafir *et al.*, 2008). These data indicate direct interference with the autochthonous microbiota of animal origin foods, inhibiting the development and

Table 3. Frequencies of Raw Milk and Soft Cheese Samples That Presented Autochthonous Lactic Acid Bacteria with Antagonistic Activity Against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Lactobacillus sakei*

Target microorganism	*Raw milk,* n *(%)*	*Soft cheese,* n *(%)*
Listeria monocytogenes	16 (44.4)	11 (61.1)
Staphylococcus aureus	5 (13.8)	2 (11.1)
Salmonella Typhimurium	0 (0)	0 (0)
Lactobacillus sakei	12 (33.3)	8 (44.4)
Total	36	18

n, number of samples.

Table 4. Frequencies of Autochthonous Lactic Acid Bacteria Isolated from Raw Milk and Soft Cheese Samples That Presented Antagonistic Activity Against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Lactobacillus sakei*

Target microorganism[a]			*Frequency* (n)		
Lactobacillus Sakei	Listeria monocytogenes	Staphylococcus aureus	*Raw milk*	*Soft cheese*	*Total* (n)
+	−	−	4	0	4
+	+	−	22	18	40
+	+	+	12	3	15
−	+	+	0	0	0
−	−	+	2	0	2
+	−	+	1	0	1
−	+	−	14	6	20

[a]Pattern of observed sensitivity.
+, inhibition; −, inhibition absence; *n*, number of cultures that presented the specific sensitivity pattern.

isolation of pathogens, as proposed by Jay (1995, 1996). Despite this relation between the autochthonous microbiota and the direct interference on some foodborne pathogens, it is important to stress the need for good producing practices during the obtaining and processing of milk and dairy products, to provide high quality and safety to these products. However, as the microbiological quality of milk improves, low counts would be observed and less competition or antagonistic substances of the foodborne pathogens observed, indicating the necessity of high hygienic control to keep the microbiological quality and safety of milk and dairy products.

The participation of LAB in dairy products microbiota can be considered relevant since these microorganisms are naturally present in milking and processing environments, facilitating the contamination of raw milk and processed products (Casalta and Montel, 2008; Franciosi *et al.*, 2009). The obtained results confirm LAB contribution to the autochthonous microbiota of the analyzed samples, since 34 (94.4%) and 15 (83.3%) samples of raw milk and soft cheese presented counts higher than 10^3 CFU/mL and 10^5 CFU/g (Table 1). In addition, LAB counts showed higher and significant indices of correlation with hygiene indicator microorganisms (Table 2), indicating that this group contributes in a direct and positive relation to the microbiota of raw milk and cheese (López-Díaz *et al.*, 2000; Han *et al.*, 2007). Despite the potential of acidification and spoilage in milk and dairy products (Galia *et al.*, 2009), distinct species and strains of LAB are able to produce different substances with antimicrobial activity (Riley and Wertz, 2002; Ross *et al.*, 2002). In such context, these substances produced by LAB are able to inhibit the development of foodborne pathogens eventually present in food. Particularly in milk, the spoilage effect due to acid production can quickly create an unsatisfactory environment that is inadequate for the survival of pathogens. The relevant presence of antagonistic LAB strains (Tables 3 and 4) associated with the high counts of hygiene indicator microorganisms (Table 1) indicates the ability of naturally occurring LAB to interfere with the growth of Gram-positive foodborne pathogens, as suggested by Jay (1995, 1996) and Nero *et al.* (2008).

Antimicrobial activity of naturally occurring LAB from animal-based products has been previously described by several authors, with results similar to the present study. Benkerroum *et al.* (2000) found high frequencies of LAB isolated from several foods, including dairy products, with antimicrobial activity against pathogens, mainly *L. monocytogenes*. Coventry *et al.* (1997) showed that milk and dairy products presented high percentage of bacteriocinogenic LAB against *L. monocytogenes* 4A and *St. aureus*. Jones *et al.* (2008), Dàvila *et al.* (2006), and Schillinger and Lücke (1989) isolated several LAB strains from meat products and verified that they presented antagonistic activity against one or more target pathogen. The absence or low frequency of LAB antagonistic against Gram-negative pathogens has been described previously (Coventry *et al.*, 1997; Schillinger and Lücke, 1989; Bromberg *et al.*, 2006; Castellano *et al.*, 2008; Nero *et al.*, 2008) and can be explained by the presence of a double lipid coating that inhibits the interaction between the antagonistic substances, such as bacteriocins, and the microorganism (Cotter *et al.*, 2005). However, LAB can play an important role against Gram-negative pathogens due to the production of organic acids, hydrogen peroxide, or reuterin, or through competitive exclusion mechanisms (Ross *et al.*, 2002; Dodd *et al.*, 2007; D'Amico *et al.*, 2008).

The ability of autochthonous LAB in producing antimicrobial substances can be a plausible reason for the absence of *L. monocytogenes*, as well the low incidence of *St. aureus*, as suggested by Topisirovic *et al.* (2006). In addition to the direct interference in these foodborne pathogens in the foods, the autochthonous LAB can also hinder the multiplication and isolation of foodborne pathogens during the enrichment steps of conventional isolation procedures (Vlaemynck and Moermans, 1996; Suh and Knabel, 2001; Nero *et al.*, 2008). This activity can also explain the negative results obtained in the present study. However, the low level of contamination of these foodborne pathogens in the milking environment cannot be disregarded. As occurring in foods, LAB and other microorganisms that are naturally present in the production and processing environment can inhibit other microorganisms, such as foodborne pathogens, reducing the chances of contamination (Holzapfel *et al.*, 2001).

The obtained results indicated that the autochthonous LAB from raw milk and raw milk cheese can interfere significantly the survival of foodborne pathogens, with concomitant compromising of the microbiological quality of these products. Despite the spoilage potential, the LAB present in the studied food products can be isolated and properly characterized as antagonistic cultures, to be used themselves or their antimicrobial products in bioconservation studies for pathogen control in foods.

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No competing financial interests exist.

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