ORIGINAL ARTICLE

Listeria monocytogenes and *Salmonella* spp. in Raw Milk Produced in Brazil: Occurrence and Interference of Indigenous Microbiota in their Isolation and Development

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Impacts

- Occurrence of foodborne pathogens in milk produced in developing country (Brazil). This occurrence differs from developed countries, like United States or European countries, which can be observed a higher prevalence of foodborne pathogens in the same food products. The differences founded in these countries, specially concerning production practices, can interfere in the survival of these foodborne pathogens.
- Conventional pathogen isolation methodologies. The obtained results lead to a discussion about the efficiency of the conventional methodologies for foodborne detection in foods with high levels of autochthonous microbiota. It became clear the interference of the autochthonous microbiota in the survival of artificially added pathogens in milk, represented mainly by Lactic Acid Bacteria (LAB). This group as identified as naturally occurring in raw milk, and also presented antagonistic activity against *Listeria monocytogenes* and *Salmonella* spp. Considering these facts, the presence of occurring LAB in milk can create inadequate conditions for pathogens survival, in milk itself or even during the isolation steps.
- A global forum about the results can be conducted in order to verify the reasons for lower levels of pathogens isolation in developing countries, associated to higher levels of microbiological contamination in the food samples, in contrast with the higher levels of the same pathogens isolation in developed countries. The autochthonous microbiota of foods can play an important role in pathogen destruction, interfering in the isolation procedures still.

Keywords:
Milk; *Listeria monocytogenes*; *Salmonella* spp.; methodology; antagonism

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Summary

This study aimed to verify the occurrence of *Listeria monocytogenes* and *Salmonella* spp. in raw milk produced in Brazil. On account of the poor microbiological quality of this product, possible interference from the indigenous microbiota in these pathogens was also evaluated. Two-hundred and ten raw milk samples were collected in four important milk-producing areas in Brazil, tested for *L. monocytogenes* and *Salmonella* spp. presence, and for enumeration of indicator microorganisms: mesophilic aerobes, total coliforms and *Escherichia coli*. The interference of the indigenous microbiota in the isolation procedures was also tested, as well the frequency of naturally occurring raw milk strains with antagonistic activity against both pathogens. The pathogens were not isolated in any raw milk sample, but poor microbiological quality was confirmed by the high levels of indicator microorganisms. When present at high levels, the indigenous microbiota generated an evident interference in the

methodologies of *L. monocytogenes* and *Salmonella* spp. isolation, mainly when the pathogens appeared at low levels. Three-hundred and sixty raw milk strains were tested for antagonistic activity against both pathogens, and 91 (25.3%) showed inhibitory activity against *L. monocytogenes* and 33 (9.2%) against *Salmonella* spp. The majority of the antagonistic strains were identified as Lactic Acid Bacteria species, mainly *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium*, known by antimicrobial substance production.

Introduction

Despite the probabilities of raw milk being a potential carrier of food-borne pathogens, some studies have shown low frequencies of these microorganisms in this product and its derivates (Carlos et al., 2001; Dhanashree et al., 2003; Franco et al., 2003; Chye et al., 2004). A frequent concomitant finding is the poor microbiological quality of the samples, which presents high levels of indicator microorganisms, such as mesophilic aerobes and coliforms (Aygun and Pehlivanlar, 2006; Kivaria et al., 2006; Adesiyun et al., 2007). These microorganisms may play an important role in the raw milk microbiota ecology, causing some interference in the development of eventual pathogens, as suggested by Jay (1995, 1996). It is well known that pathogens need specific conditions to survive and multiply, and any change in their substrate can hinder their growth (Jay et al., 2005).

The indigenous microbiota of raw milk is composed by different groups of microorganisms, and Lactic Acid Bacteria (LAB) is an important one. LAB can produce different substances with antimicrobial activity, such as organic acids, hydrogen peroxide, diacetyl and bacteriocins (Riley and Wertz, 2002; Ross et al., 2002). On account of their development, LAB can hinder the growth of pathogens in the food itself, or even during the laboratorial procedures of pathogen recovery, when enrichment phases are required (de Boer, 1998; Jiang et al., 1998).

Considering the importance of recognizing microbial hazards in foodstuffs, according to the first step of risk assessment, this study aimed to detect *Listeria monocytogenes* and *Salmonella* spp. in raw milk produced in Brazil. As this food has a notoriously poor microbiological quality, possible interference of the raw milk microbiota in these pathogens and their isolation methodologies were also researched.

Materials and Methods

Occurrence of *L. monocytogenes* and *Salmonella* spp. and microbiological quality

Two-hundred and ten raw milk samples were collected in four important milk-producing States in Brazil: Minas Gerais (47 samples, Viçosa region), Rio Grande do Sul (50, Pelotas region), Paraná (63, Londrina region) and São Paulo (50, Botucatu region) (Fig. 1). All samples were submitted to *L. monocytogenes* and *Salmonella* spp. detection according APHA (Wehr and Frank, 2004), using culture media from Oxoid (Oxoid Ltd., Basingstoke, England).

The samples were also diluted serially in a decimal scale in 0.85% NaCl for enumeration of indicator microorganisms (mesophilic aerobes, total coliforms and *Escherichia coli*) using Petrifilm™ AC and EC plates (3M Company, St Paul, MN, USA), incubated at 35°C for 48 h, according to manufacturer's guidelines.

Strains

Listeria monocytogenes ATCC 7644 and *Salmonella* Enteritidis ATCC 13076 were kept in Trypticase Soy Agar (TSA) (Oxoid) slants at 4°C. At the moment of use, the

Fig. 1. Milk producing regions from Brazil where 210 raw milk samples were collected.

strains were streaked in TSA plates and incubated at 30–35°C for 24 h, when a single colony was transferred to Trypticase Soy Broth (TSB) (Oxoid) and incubated at 30–35°C for 24 h. After turbidity of the medium, an aliquot of the culture was analyzed for absorbance (λ = 660 nm) to estimate the pathogen concentration.

Interference of indigenous microbiota over pathogen isolation methodologies

Three samples of raw milk were used to evaluate the performance of the *L. monocytogenes* isolation methodology and three for *Salmonella* spp. Initially the samples were diluted twice in reconstituted powdered skimmed milk (1 : 10, Molico™; Nestlé, São Paulo, Brazil) to obtain different levels of contamination by the indigenous microbiota. Each one of the raw milk samples and their two dilutions were then subdivided into four 100 ml aliquots, which were experimentally contaminated with *L. monocytogenes* or *S.* Enteritidis cultures to contain different concentrations of each pathogen.

The reconstituted powdered skimmed milk used for dilution was used as control, and also subdivided into four 100 ml aliquots that were inoculated with the same cultures. The controls were diluted serially in decimal scale in 0.85% NaCl and grown on Petrifilm™ AC incubated at 35°C for 48 h to determine the exact concentration of the pathogen.

Immediately after inoculation, the treatments inoculated with *L. monocytogenes* were submitted to the official detection methodology for this pathogen, and similar procedures were conducted with the treatments inoculated with *S.* Enteritidis (Wehr and Frank, 2004).

Before the experimental inoculation, the raw milk samples and their dilutions were grown on Petrifilm™ AC, incubated at 35°C for 48 h, to determine the concentration of the indigenous microbiota. After the microbiological analysis, the results of all treatments were expressed as positive or negative for *L. monocytogenes* or *S.* Enteritidis, associated with the level of pathogen and indigenous microbiota contamination.

Detection of antagonistic indigenous microbiota

Fifteen raw milk samples were submitted to serial decimal dilutions in 0.85% NaCl and plated by surface on Plate Count Agar (PCA) (Oxoid), with incubation at 35°C for 48 h. For each milk sample, twenty-four isolated colonies were randomly selected and spotted simultaneously onto five replicate plates containing PCA and incubated at 35°C for 24 h. Two PCA plates were used to test for antagonistic activity against *L. monocytogenes* and two for antagonistic activity against *S.* Enteritidis, using the 'spot-on-the-lawn' methodology (Lewus and Montville, 1991). A total of 360 colonies were tested. When antagonism was detected, the corresponding colonies on the remaining PCA plates were submitted to identification by Gram stain, catalase test and biochemical profile by API 20 Strep (bioMérieux S.A., Lyon, France).

Results

Occurrence of *L. monocytogenes* and *Salmonella* spp. and microbiological quality

All tested samples were negative for *L. monocytogenes* and *Salmonella* spp. The frequencies of the different levels of contamination by the researched indicator microorganisms are shown in Table 1. It is possible to observe the poor microbiological quality of the tested samples on account of the high frequency of samples with elevated levels of contamination.

Interference of indigenous microbiota over pathogen isolation methodologies

The inoculated pathogen was recovered in all the treatments carried out with reconstituted powdered skimmed milk artificially contaminated with *L. monocytogenes* or *S.* Enteritidis, regardless of their concentration. Analyzing the results obtained from the treatments, the recovering of the inoculated pathogen did not occur in treatments with high levels of indigenous microbiota associated with low levels of the pathogen (Fig. 2). This interference was more evident in the treatments inoculated with *L. monocytogenes*.

Table 1. Frequencies of raw milk samples collected in Brazil with different levels of contamination by mesophilic aerobes, total coliforms and *Escherichia coli*

Intervals of contamination (log CFU/ml)	Minas Gerais, *n* (%)	Rio Grande do Sul, *n* (%)	Paraná, *n* (%)	São Paulo, *n* (%)	Total, *n* (%)
Mesophilic aerobes					
≤3	2 (4.3)	0 (0.0)	2 (3.2)	0 (0.0)	4 (1.9)
3–5	20 (42.6)	9 (18.0)	13 (20.6)	3 (6.0)	45 (21.4)
>5	25 (53.2)	41 (82.0)	48 (76.2)	47 (94.0)	161 (76.6)
Total coliforms					
≤2	15 (31.9)	11 (22.0)	12 (21.1)	2 (4.0)	40 (19.6)
2–4	20 (42.6)	29 (58.0)	16 (28.1)	24 (48.0)	89 (43.6)
>4	12 (25.5)	10 (20.0)	29 (50.9)	24 (48.0)	75 (36.8)
Escherichia coli					
≤1	31 (66.0)	41 (82.0)	35 (61.4)	24 (48.0)	131 (64.2)
1–3	7 (14.9)	8 (16.0)	14 (24.6)	14 (28.0)	43 (21.1)
>3	9 (19.1)	1 (2.0)	8 (14.0)	12 (24.0)	30 (14.7)

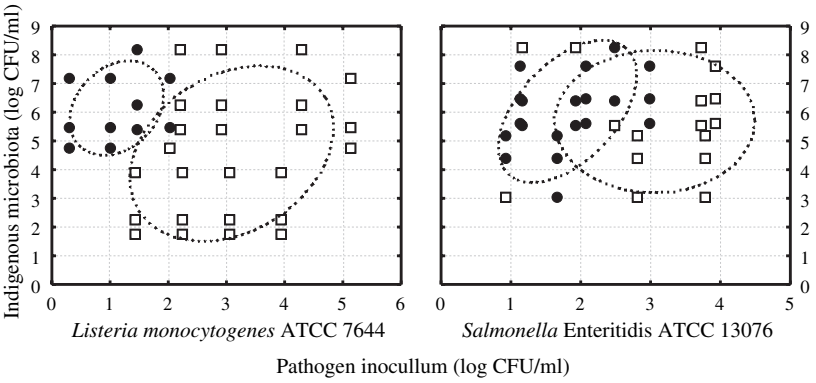

Fig. 2. Dispersion of results for recovering (□) and not recovering (●) *Listeria monocytogenes* (left) or *Salmonella* Enteritidis (right), artificially inoculated in raw milk treatments with different levels of indigenous microbiota.

Detection of antagonistic indigenous microbiota

Two types of inhibition were observed: a partial one, characterized by a diffuse halo around the antagonistic colony, and a total one, characterized by a well-defined halo. From the 360 tested colonies, 91 (25.3%) showed antagonistic activity against *L. monocytogenes*, with 80 (22.2%) presenting partial inhibition and 11 (3.1%) total inhibition. The frequency of colonies with antagonistic activity against *S.* Enteritidis was lower: 33 (9.2%) colonies showed this activity, all characterized by a partial inhibition. The results of the initial characterization of the antagonistic colonies are shown in Table 2, and it is possible to observe the predominance of Gram positive cocci and negative catalase that were identified in most of the cases as *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* using API 20 Strep strips (Table 3).

Discussion

According to the obtained results, *L. monocytogenes* and *Salmonella* spp. cannot be considered relevant hazards (Notermans et al., 1998; Codex Alimentarius Commission, 1999) in raw milk produced in Brazil. However, the samples presented poor microbiological quality, which reflects unsatisfactory hygienic conditions of milk production. When mesophilic aerobes and coliforms are present in raw milk in levels up to 5 and 2 log CFU/ml, respectively, there is evidence of serious deficiencies in production hygiene and unsatisfactory production practices (Chambers, 2002). In the raw milk samples, mesophilic aerobes occurred at this level in 76.6%, and coliforms in 80.4% (Table 1). São Paulo was the State that presented the poorest microbiological quality, whereas Minas Gerais presented better results. It should also be noted that raw-milk samples from Rio Grande do Sul presented the lowest counts of *E. coli*, indicating better hygienic conditions (Table 1).

Despite the evidence of failures in milk production in Brazil, the high levels of indigenous microbiota may have also exerted some interference in the survival or development of potential *L. monocytogenes* or *Salmonella* spp. presented in the samples. This interference could have occurred in the raw milk itself, or even during the isolation procedures. Jay et al. (2005) and Jay (1995, 1996) suggest that high levels of contamination by the indigenous microbiota of foods interferes directly in the survival and development of pathogens, as they need highly specific conditions to grow. Therefore, pathogens present in food at low levels, associated with high levels of indigenous contamination, inhibit their development.

These interactions between pathogens and indigenous microbiota are supported by similar results in studies with animal-based products with poor microbiological quality, which shows low incidence of pathogens (Kozak et al., 1996; Lopez and Sánchez, 2000; Cordano and

Table 2. Gram stain characteristics and catalase reaction of raw milk strains with partial and total inhibitory activity against *Listeria monocytogenes* and *Salmonella* Enteritidis

	Inhibitory activity against *L. monocytogenes*		Inhibitory activity against *S.* Enteritidis	
Characteristics	Total, *n* (%)	Partial, *n* (%)	Total, *n* (%)	Partial, *n* (%)
Gram positive cocci, negative catalase	10 (90.9)	68 (85.9)	–	31 (93.9)
Gram positive cocci, positive catalase	0 (0.0)	7 (8.8)	–	1 (3.0)
Gram positive rods	1 (9.1)	2 (2.5)	–	0 (0.0)
Gram negative rods	0 (0.0)	3 (3.8)	–	1 (3.0)
Total	11 (100.0)	80 (100.0)	–	33 (100.0)

Table 3. Prevalence of species isolated from raw milk, presenting inhibition against *Listeria monocytogenes* and *Salmonella* Enteritidis

Target pathogen	Type of inhibition	Inhibitory species	*n* (%)
Listeria monocytogenes	Total	*Lactococcus lactis* subsp. *lactis*	4 (44.4)
		Enterococcus faecium	5 (55.6)
		Total	11
	Partial	*Lactococcus lactis* subsp. *lactis*	18 (56.3)
		Lactococcus lactis subsp. *cremoris*	1 (3.1)
		Enterococcus faecium	7 (21.9)
		Enterococcus faecalis	2 (6.3)
		Enterococcus durans	1 (3.1)
		Streptococcus mutans	1 (3.1)
		Streptococcus salivarius subsp. *salivarius*	1 (3.1)
		Total	32
Salmonella Enteritidis	Total	None	0
	Partial	*Lactococcus lactis* subsp. *lactis*	11 (64.7)
		Enterococcus faecium	4 (23.5)
		Enterococcus durans	1 (5.9)
		Non-identified	1 (5.9)
		Total	17

Rocourt, 2001; Dhanashree et al., 2003). However, when a better microbiological quality is observed, with low levels of indigenous microbiota, the frequency of pathogens in foods tends to be higher (Loncarevic et al., 1995; Gaya et al., 1998; de Buyser et al., 2001; Guerra et al., 2001; Rudolf and Scherer, 2001; Leclerc et al., 2002).

The interference of the raw milk indigenous microbiota over *L. monocytogenes* and *Salmonella* spp. can be confirmed by the results shown in Fig. 2. It can be seen that both methodologies were inefficient for recovering trace amounts of the pathogens, when associated with high levels of indigenous microbiota in the raw milk treatments. For *L. monocytogenes* the interference was limited to specific levels for both microbial loads (pathogen and indigenous microbiota), suggesting a restricted sensibility of the methodology (Fig. 2). For *S.* Enteritidis the interference was not well evident, as in some treatments, it was possible to recover the pathogen even in low levels, associated with high levels of indigenous microbiota.

Several factors can affect the efficiency of the conventional methodologies for pathogen detection in foods. Low concentrations of the pathogens in the food samples is pointed out as an important issue (Vlaemynck and Moermans, 1996; Jiang et al., 1998; Suh and Knabel, 2001), and also the indigenous microbiota of the samples can interfere mainly in the initial phases of detection, generating unfavourable conditions for pathogen survival or detection (Busse, 1995; Vlaemynck and Moermans, 1996; de Boer, 1998; Jiang et al., 1998). In these phases, the indigenous microbiota of the food sample can multiply to exponential levels, causing a quick reduction of pH of the culture medium and, consequently, inhibiting the development of the target pathogen, compromising its detection (Jiang et al., 1998).

Microorganisms from *Enterococcus* and *Lactobacillus* genera, usually present in raw milk, have been described as important inhibitors of *L. monocytogenes*, interfering directly in the conventional isolation methodology (Vlaemynck and Moermans, 1996; Jiang et al., 1998; Suh and Knabel, 2001). For *Salmonella* spp. detection in milk, the interference of indigenous microbiota is considered unpredictable as well the interactions between this microbiota and the pathogen, and previous knowledge of its composition can help in choosing its specific and appropriate culture media (Busse, 1995). However, it is practically impossible to predict the microbiological profile of raw milk produced in Brazil.

This variable profile of raw milk microbiota was evident in the antagonistic study. There was no pattern in the frequency of antagonistic strains of the 15 raw milk samples tested. Despite this variability, two types of antagonisms were detected, probably on account of different paths of inhibition. The total inhibition could occur on account of specific interactions between the pathogen and the antagonistic production, typically from bacteriocins (Riley and Wertz, 2002; Liu et al., 2004). Other antagonistic substances can produce unspecific interactions, resulting in the observed partial inhibition, typically from organic acids and hydrogen peroxide (Ross et al., 2002). The initial characterization of these antagonistic substance producers revealed that the major part of these microorganisms are Gram positive cocci, catalase negative (Table 2). These characteristics are typical for the LAB group, and the identification of species by API 20 Strep confirmed these results (Table 3).

The antagonistic activity of LAB against pathogens, such as *L. monocytogenes* and *Salmonella* spp., has been described in milk and dairy products (Issa and Ryser, 2000; Giraffa, 2003; Heikkilä and Saris, 2003). However, this activity of the indigenous raw milk microbiota has not been described for the isolation methodologies and the pathogens eventually present in this food. The strains that presented total inhibition were *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium*, known for their bacteriocin-producing ability with activity more specific against Gram positive pathogens, such as *L. monocytogenes* and *Staphylococcus aureus* (Riley and Wertz, 2002; Franz et al., 2003; Liu et al., 2004). The obtained results confirm these findings, as only *L. monocytogenes* showed

sensibility to the strains with total inhibitory activity, probably on account of bacteriocin production.

The naturally occurring raw milk LAB with antagonistic activity against *L. monocytogenes* and *Salmonella* spp. could have generated unsatisfactory conditions for survival of these pathogens during the initial steps of isolation or even in the raw milk itself. For these reasons, these pathogens cannot be considered hazards present in raw milk, as they are typically considered in some conditions. The interactions between this raw milk antagonistic microbiota and the main target pathogens associated with this product must be clarified by proper identification of the inhibitory substances and their microorganism producers, aiming to elucidate the natural protection that raw milk microbiota confers to this product.

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