10.4322/rbcv.2022.027

Potentially pathogenic *Staphylococcus aureus and Listeria spp.* in Brazilian unpasteurized cheese production

Staphylococcus aureus e *Listeria spp.* potencialmente patogênicos na produção de queijos não pasteurizados

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Abstract

This study aimed to identify potentially pathogenic microorganisms (*Listeria innocua, L. seeligeri, L. ivanovii, L. monocytogenes*, *Staphylococcus aureus,* and several virulence genes) in unpasteurized cheese production in the northeastern region of the state of São Paulo, Brazil. *Listeria* species were detected in 68 (64.14%) out of 106 samples of bovine feces, swabs from milkers' and cheese handlers' hands, milking buckets, raw milk, whey, water, cheese processing surface,s and utensils. All the samples collected at one farm were contaminated with *Listeria* spp. *L. innocua, L. seeligeri, L. ivanovii,* or *L. monocytogenes* were not detected in the samples collected in this study. A set of 391 *Staphylococcus* spp. isolates were obtained in these samples, from which 60 (15.31%) were identified as *S. aureus* using PCR (Polymerase Chain Reaction). *S. aureus* carrying virulence genes (*eta*, *hlg*, *seg*, *seh*, *sei*) were detected in milk, in swabs from cheese handler's hands, whey, milk, sieves, buckets, and cheese. The *hlg* gene (encodes gamma hemolysin) was detected in all the *S. aureus* isolates. These findings show that poor hygienic practice is associated with a higher risk of pathogenic bacteria in milk or cheese, providing useful information for public health authorities to increase food safety surveillance and prevent the dissemination of pathogens.

Keywords: bovine milk, contaminated food, enterotoxin, public health.

Resumo

O objetivo desse estudo foi identificar microrganismos potencialmente patogênicos (*Listeria innocua, L. seeligeri, L. ivanovii, L. monocytogenes*, *Staphylococcus aureus* e diversos genes de virulência) na produção de queijos de leite cru na região noroeste do Estado de São Paulo, Brasil. *Listeria* foram detectadas em 68 (64,14%) das 106 amostras obtidas de fezes bovinas, suabes das mãos de ordenhadores e queijeiros, baldes, leite cru, soro, água, superfícies e utensílios da produção de queijos. Todas as amostras coletadas em uma fazenda estavam contaminadas com *Listeria* spp. *L. innocua, L. seeligeri, L. ivanovii,* e *L. monocytogenes* não foram detectadas nas amostras coletadas nesse estudo. Um conjunto de 391 isolados de *Staphylococcus* spp. foram obtidos das amostras, e desses 60 (15,31%) foram identificados como *S. aureus* pela PCR (*Polymerase Chain Reaction*). *S. aureus* contendo genes de virulência (*eta*, *hlg*, *seg*, *seh*, *sei*) foram detectados em leite, mãos dos ordenhadores, soro, utensílios e queijos. O gene *hlg* (gama-hemolisina) foi detectado em todos os isolados de *S. aureus.* Esses resultados demonstram que práticas inadequadas de higiene estão associadas com um maior risco da presença de bactérias patogênicas no leite e queijos crus, fornecendo informações para as autoridades de saúde pública para incrementarem a vigilância e prevenirem a disseminação de patógenos.

Palavras-chave: alimentos contaminados, enterotoxina, leite bovino, saúde pública.

Introduction

Unpasteurized cheeses are widely illegally sold in Brazil. Artisanal unpasteurized cheese is produced in a traditional process andis the most widely consumed in the country (about 30 g/person/day) (IBGE, 2011).

Nevertheless, both raw and pasteurized cheeses have been involved in several foodborne outbreaks. The most frequent pathogens associated with dairy products are strains of *Staphylococcus aureus*, *Salmonella* spp., *Listeria*

*Recebido em 19 de outubro de 2022 e aceito em 18 de novembro de 2022.

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monocytogenes, Brucella spp*, Campylobacter jejuni, Bacillus cereus, Mycobacterium tuberculosis, Mycobacterium bovis,* and pathogenic *Escherichia coli* (Dhanasheka, et al., 2012).

L. monocytogenes affects mainly the elderly, newborns, pregnant women and immunocompromised people (Schlech., 2019). The main symptoms of listeriosis infection vary from flu and gastroenteritis to meningitis, encephalitis, miscarriage, premature birth or stillbirth. The lethality of this infection is comparable to that of encephalitis (Matle el al., 2020). Another important bacterium is *Staphylococcus* spp. The main symptoms of staphylococcal food poisoning are nausea, vomiting, abdominal cramps, diarrhea, sweating, headaches and, in some cases, feverish or hypothermic conditions (Silva et al., 2020).

These microorganisms are mainly transmitted through the ingestion of unpasteurized dairy products. The general lack of awareness of milk-borne infections and several traditional practices poses consumers of milk and dairy products at a high risk of illness (Chengat Prakashba et al., 2020).

The poor sanitary and hygiene conditions in which artisanal cheeses are produced in Brazil have been described (Nunes et al., 2013), however, this problem remains a risk for public health. Thus, this study focused on detecting the pathogenic potential of *Listeria* spp. and *S. aureus* strains identified in several stages of the production chain of unpasteurized cheese in Brazil to provide information for public health authoritiestoo improve food safety and protect public health in Brazil.

Material and methods

During 2014, a set of samples were collected of bovine feces, swab samples from milkmen's and cheese handlers' hands, from milking buckets, remove samples of unpasteurized artisanal cheese, whey, water, cheese processing surfaces, sieves, trays, molds, and skimmers at five non-technified dairy farms (A, B, C, D, E) in the northeastern region of the state of São Paulo, Brazil. All material was collected and stored in sterile material and hands were previously sanitized with 70% alcohol. The cheeses were stored in sterile bags, as the producers did not pack them in advance (Brito et al., 1998; Alpha el al., 2001). All material was transported in isothermal boxes containing ice and taken to the Laboratory of Analysis of Food of Animal Origin and Water, located in the Department of Preventive Veterinary Medicine and Animal Reproduction, of the Faculty of Agricultural and Veterinary Sciences of Jaboticabal (FCAV-UNESP), where they were processed immediately. At least 17 samples (one of each type of sample, two cheeses, and five bovine feces samples) were collected at each farm, making a total of 106 samples, 22 from farm A, 23 from B, 21 from C, 21 from D, and 19 from E. All these samples were collected in tubes containing 6mL of peptone water 0.1%.

All farms had manual milking in which the milk was collected in buckets. Room milking was open (without walls, in contact with the environment), with dirt from soil particles and animal feces. There was no pre-dipping and post-dipping and producers did not carry out water quality analysis. In addition, the utensils used for milking and cheese production were also left in the dust. Also, cheese production does not have standardized hygiene. The person that did the milking at each farm was also the one directly involved in cheese making.

Tubes containing swabs and peptone water (including those containing bovine feces) were homogenized in the laboratory by vortexing. Then, 1 mL of each sample was placed in a vial containing 5 mL of Listeria enrichment broth (LEB). The isolation protocol specified by the United States Department of Agriculture was followed (USDA, 1996). Water samples were filtered through a sterile membrane filter, 47 mm in diameter with 0.45 µm pore size (Vicente et al., 2005), and then poured into vials containing 50 mL of LEB.

Secondary selective enrichment was then performed after incubation in Fraser broth (supplemented for *L. monocytogenes*). Part of the culture was then inoculated in Petri dishes containing modified Oxford agar (MOX) (USDA, 1996). In addition, three to five typical colonies of *Listeria* spp. were inoculated in tubes containing Brain Heart Infusion (BHI broth, USDA, 1996), after which the DNA was extracted as described by Keskimaki et al. (2001).

Listeria spp. isolates were confirmed by PCR using the oligonucleotide pair *DG75* and *DG76* (Choi; Hong, 2003). The species *L. monocytogenes* was identified using the oligonucleotides *mono A* and *Lis1B* (Bubert et al., 1999). Multiple virulence factors, including phosphatidylinositol phospholipase C (*plcA*), hemolysin (*hlyA,*) and invasive associated protein (*iap*) are necessary for the pathogenesis of *L. monocytogenes* (Rawool *et al*., 2007) and these three virulence factors encoding genes *plcA*, *iap* and *hly* were identified using the oligonucleotide primers developed by Rantsiou et al. (2012). Isolates negative for *L. monocytogenes* were subjected to PCR to detect *L. innocua, L. seeliger,i* and *L. ivanovii* species (Bubert et al., 1999), which are known to cause human infections (Table 1).

Tubes containing swabs and peptone water (including those containing bovine feces) were homogenized in the laboratory by vortexing. Then, 1 mL of this solution was added to vials containing 5 mL of BHI broth. All the samples were incubated at 37° C for 24 hours (APHA, 2001). Water samples were filtered through a sterile membrane filter, 47 mm in diameter with 0.45 µm pore size (Vicente et al., 2005), and then poured into vials containing 50 mL of BHI.

One loopful of each sample cultured in BHI broth was inoculated in Petri dishes containing Baird Parker agar. In addition, three colonies typical of *Staphylococcus* spp. (black, shiny, convex, surrounded by a clear area and may have an opaque border) from each sample were inoculated in tubes containing BHI broth (USDA, 1996). After incubation, smears were prepared and stained by the Gram method, after which positive isolates were subjected to free coagulase tests (MacFaddin, 1976).

DNA was extracted from all the isolates typical of *Staphylococcus* spp., Gram-positive cocci and free coagulase-positive samples, as described by Keskimaki et al. (2001). Then, a PCR assay was performed for genus confirmation (*16s RNA*, according to Pereira et al., 2009) and *S. aureus* species detection (*Sa442*, according to Morot-Bizot et al., 2004). Isolates identified as *S. aureus* were tested for the presence of the *coa* gene for coagulase-positive (Blickwede et al., 2005) and for the presence of toxin encoding genes (*sea, seb, sec, sed, see, seg, seh, sei, tst, eta, pv,l* and *hlg*, as described by Paniagua-Contreras et al. (2012) (Table 2).

Table 1: The sequencee of oligonucleotide primers to identify *Listeri*a spp., *L. monocytogenes, L. innocua,L. seeligeri* and *L. ivanovii*, and the three genes encoding virulence factor for *L. monocytogenes plcA, iap* and *hly*, size, amplification product, temperature, positive contro, l and their respective reference

	Oligonucleotide primers	Sequence	Size (bp)	Temperature (°C)	Positive control	Reference
Listeria spp	*DG75*	GACCGCAAGGTTGAAACTCA	421	60	ATCC19114	Choi; Hong, 2003
	DG76	CAGCCTACAATCCGAACTGA				
L. monocytogenes	*monoA*	CAAACTGCTAACACAGCTACT	660	58	ATCC19114	Bubert et al., 1999
	Lis1B	TTATACGCGACCGAAGCCAAC				
L. ivanovii	*iva1*	CTACTCAAGCGCAAGCGGCAC	1100	58	IAL 1983	
	Lis1B	TTATACGCGACCGAAGCCAAC				
L. innocua	*ino2*	ACTAGCACTCCAGTTGTTAAAC	870	58	ATCC 33090	
	Lis1B	TTATACGCGACCGAAGCCAAC				
L. seeligeri	*Sel1*	TACACAAGCGGCTCCTGCTCAAC	1100	58	IAL 2370	
	Lis1B	TTATACGCGACCGAAGCCAAC				

Table 2: Sequence of oligonucleotide primers for the identification of genus *Staphylococcus*, species *S. aureus* and enterotoxin-encoding genes, *sea, seb, sec, sed, see, seg, seh, sei, tst, eta, pvl and hlg,* amplification of *S. aureus* product size, annealing temperature, positive contr,ol and their respective reference

	Oligonucleotide primers	Sequence	Size (bp)	Annealing temperature (°C)	Positive control	Reference
Staphylococcus spp	*16sRNA*	GTA GGT GGC AAG CGTTAT CC CGC ACA TCA GCG TCA G	228		ATCC25923	Pereira et al., 2009
S. aureus	*Sa442*	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA	102	55	ATCC25923	Morot-Bizot et al., 2004
S. aureus	*TstaG422/765*	GGCCGTGTTGAACGTGGTCAAATCA TIACCATTTCAGTACCTTCTGGTAA	370		ATCC25923	Morot-Bizot et al., 2004
Coagulase encoding gene of *S. aureus*	*coa*	AGAAGGTCTTGAAGGTAGC GAATCTTGGTCTCGCTTC	250	55	ATCC6538	Blickwede et al., 2005
Genes that encode toxin proteins from *S. aureus*	*sea*	TTGCAGGGAACAGCTTTAGGCAATC TGGTGTACCACCCGCACATTGA	252	60	ATCC23235	Paniagua-Contreras et al., 2012
	seb	GACATGATGCCTGCACCAGGAGA AACAAATCGTTAAAAACGGCGACACAG	355	60	ATCC14458	
	sec	CCCTACGCCAGATGAGTTGCACA CGCCTGGTGCAGGCATCATATC	602	60	ATCC19095	
	sed	GAAAGTGAGCAAGTTGGATAGATTGCGGCTAG CCGCGCTGTATTTTTCCTCCGAGAG	830	60	ATCC23235	
	see	TGCCCTAACGTTGACAACAAGTCCA TCCGTGTAAATAATGCCTTGCCTGAA	532	60	ATCC27664	
	seg	TGCTCAACCCGATCCTAAATTAGACGA CCTCTTCCTTCAACAGGTGGAGACG	117	60	ATCC25923	

	Oligonucleotide primers	Sequence	Size (bp)	Annealing temperature (°C)	Positive control	Reference
Genes that encode toxin proteins from *S. aureus*	*seh*	CATTCACATCATATGCGAAAGCAGAAG GCACCAATCACCCTTTCCTGTGC	358	60	ATCC19095	Paniagua-Contreras et al., 2012
	sei	TGGAGGGGCCACTTTATCAGGA TCCATATTCTTTGCCTTTACCAGTG	220	60	ATCC19095	
	tst	AGCCCTGCTTTTACAAAAGGGGAAAA CCAATAACCACCCGTTTTATCGCTTG	306	60	ATCC25923	
	eta	CGCTGCGGACATTCCTACATGG TACATGCCCGCCACTTGCTTGT	676	60	TC-142	
	pvl	TGCCAGACAATGAATTACCCCCATT TCTGCCATATGGTCCCCAACCA	894	60	ATCC25923	
	hlg	TTGGCTGGGGAGTTGAAGCACA CGCCTGCCCAGTAGAAGCCATT	306	60	ATCC19095	

Results and discussion

The genus *Listeria* spp. was detected in 68 (64.14%) of a total of 106 samples. At farm A, these species were detected in 33.36% of samples, at farm B in 56.52%, at farm C in 61.90%, and at farm D in 71.43% of samples. All the samples collected at farm E were contaminated with *Listeria* (Table 3). Samples of water, swabs from milk and cheese handlers' hands, buckets, and sieves were contaminated with *Listeria* species, which may explain the presence of this genus in cheese samples. It is known that milk and cheese may be contaminated by contacting food processing surfaces (Hanson et al., 2019).

Table 3: Samples contaminated with *Listeria* spp. from five farms that produce unpasteurized cheese in the northeast region of the state of São Paulo, Brazil

	Farm				
	A	**B**	**C**	**D**	**E**
Samples positive for *Listeria spp.* / Number of Collected Samples (%)	**8/22 (33.36%)**	**13/23 (56.52%)**	**13/21 (61.90%)**	**15/21 (71.43%)**	**19/19 (100%)**
	Number of contaminated samples				
Milk	1	1	1	1	1
Bovine feces	5	5	5	5	5
Cheese	1	2	2	2	2
Mold	1	1	-	-	1
Water	-	2	-	-	-
Cheese making surface	-	1	-	1	1
Whey	-	1	1	1	1
Water from cheese making room	-	-	1	1	1
Cheese handler	-	-	1	-	1
Thermometer	-	-	1	-	-
Spoon to cur the curd	-	-	1	1	-
Milkers's hands	-	-	-	-	1
Sieves	-	-	-	2	3
Buckets	-	-	-	1	2

Foodborne pathogens cause an important public health burden, which is estimated in 600 million cases and more than 400,000 deaths, globally every year. Food safety incidents, outbreaks, sporadic cases, and recalls have recognized economic impact, estimated at 7 billion every year in the US. Food safety has become a priority, and the implementation of preventive controls and monitoring systems has raised the development of new tools to detect and prevent pathogens in the food chain (Rivera et al., 2018). Importantly, to reduce food safety hazards hygienic measures must be adopted from farm to fork and the presence of pathogens, such as *Listeria* spp. and *Staphylococcus*, must be better understood in the dairy production chain (Dhanasheka et al., 2012).

This high level of *Listeria* spp. contamination may be explained by the fact that the milking sheds at all the farms of this study have earthen floors. *Listeri*a spp. is widely distributed in nature and soil is an important habitat for these bacteria (Linke et al., 2014) and the raw milk may be contaminated with this microorganism through poor hygiene practices during milking.

Several studies have reported the presence of *Listeria* spp. in bulk milk (Soltysiuk et al., 2022) and milk from cows with mastitis (Jamali and Radmehr, 2013). Contamination by *Listeria* genus has been identified in different samples in the present study, such as bovine feces, cheese mold, cheesemaking surfaces, water, milkers' and cheese handlers' hands, thermometers, spoons, milk sieves, and milking buckets. A study in Ethiopia detected *Listeria* spp. in 28.4% of samples (60% of cheese, 40% of pasteurized milk, and 5% of yoghurt samples) (Seyoum et al., 2015). Jamali and Radmehr (2013) suggested that continuous surveillance of this genus in foods is needed due to the zoonotic potential of *Listeria* spp. and the health risks posed by milk and dairy products.

However, the pathogenic species *L. monocytogenes, L. innocua, L. seeligeri,* and *L. ivanovii* were not detected in the present study. This can be explained by the microbial competition that occurs during selective enrichment, negatively affecting populations of *L. monocytogenes* and possibly affecting the detection or recovery of this bacteria in samples (Keys et al., 2016)

The genus *Listeria* spp. was detected in a high number of samples in this study. This demonstrates that good milking and cold storage practices are essential factors to ensure milk hygiene and safety, especially in tropical countries (Ramirez-Riviera et al., 2019), such as Brazil. None of the farms sampled in this study used adequate hygiene practices during milking, although this was only visually observed and not examined systematically. The dairy farming practices generally adopted at these farms were consumption of raw milk (68.1%), local sale of raw milk (25.2%), absence of cooling facilities at small farms (98%), and absence of routine testing (84.9%) and medical check-ups (89.1%) for milk-borne zoonotic diseases. General hygiene and disease control practices should be integrated in the milk production process, particularly at the smallholder level (Hanson et al., 2019).

The most important factor is proper hygiene practices (Gwida, et al., 2020) but it is also important to have an efficient system for monitoring foods contaminated with *Listeria* spp. Surveillance integrated into the food chain through microbiological analysis is an excellent way to monitor the risk of occurrence of foodborne diseases, but this approach requires greater investment by the industry, as well as greater multisectoral collaboration with the public health system (Ford et al., 2015).

Three hundred and ninety-one *Staphylococcus* spp. isolates were obtained and the specie *Staphylococcus aureus* was confirmed in 68 isolates (17.39%), mainly from cheese and milk samples (Table 4). This may be attributed to the fact that the person who did the milking and produced cheese at this farm did not wear gloves, did not trim his fingernails, and used finger rings. The accessories favor the formation of biofilms and can carry microorganisms and consequently contaminate food (Pacha et al., 2021).

In this study, the *hlg* gene was detected in all the *S. aureus* isolates (Table 4). This gene encodes gamma hemolysin, whose proinflammatory activity can cause the lysis of leukocytes and erythrocytes (Annamanedi et al., 2021). This gene was the most frequently detected in *S. aureus* isolates from cows with intramammary infections, as well as from the skin of dairy cows, and milkers' skin lesions, hands, and nostrils (Mahato et al., 2017). Our results indicate that contamination originated in milk from cows with subclinical mastitis or milkers.

Table 4: Samples contaminated with *Staphylococcus aureus* from five farms that produce unpasteurized cheese in the northeast region of the state of São Paulo, Brazil

	A	B	C	D	E
Samples positive for *Staphylococcus aureus*/ Number of Collected Samples (%)	**3/22 (13,64%)**	**3/23 (13,04%)**	**3/21 (14,28%)**	**4/21 (19,05%)**	**6/19 (31,58%)**
Milk	1 (hlg)	-	-	2 (hlg)	2 (hlg)
Bovine feces	-	-	-	-	1(hlg)
Cheese 1	-	-	-	1 (hlg)	2 (seg, eta, sei, hlgl)
Cheese 2	2 (hlg)	3 (seh, hlg)	-	-	4 (seg, eta, sei, hlg)
Mold	-	-	-	-	-
Water	-	-	-	-	-
Cheese making surface	-	-	-	-	-
Whey	-	-	1 (seg, sei, hlg)	-	-
Water from cheese making room	-	-	-	-	-
Cheese handler	1 (hlg)	-	-	-	-
Thermometer	-	-	-	-	-
Spoon to cur the curd	-	-	-	-	-
Milkers's hands	-	-	-	-	-
Sieves	-	-	-	3 (seg, eta, sei, hlg)	3(hlg)
Buckets	-	-	-	3 (hlg)	3(seg, eta, sei, hlg)

In one study, the *hlg* gene was identified in 96.3% of *S. aureus* isolates in hemodialysis catheters from patients in Mexico, followed by 92.7% of the *seg* gene, 85.4% of the *sei* gene, and 78.1% of the *seh* gene (Paniagua-Contreras et al., 2012). In the present study, these genes were also detected in *S. aureus* isolates from unpasteurized cheeses, indicating the potential risk they represent for causing infections or food poisoning in humans.

Also, it is important to show that at farm A *hlg* was find at cheese, cheese handler, and milk. So contamination can occur from cheese handler. In farm D, cheese was contaminated with *hlg* and the same virulence factor appears in the bucket and sieve. Furthermore, at farm E cheese was contaminated with *seg, eta, sej,* and *hlg,* and *seg, eta,* and *sej* were found at bucket and *hlg* was found in milk, bovine feces, and sieve, showing that cheese contamination can be coming from these sources.

S. aureus carrying genes that encodes enterotoxins (*sea, seb, sec, sed, see, seg, she, sei, tst, eta,* and *pvl*) was widespread in several steps of the milking and cheese production process. They were detected in samples of whey, sieves, buckets, and most importantly, in the cheeses. Staphylococcal food poisoning is characterized by gastrointestinal symptoms and is the result of eating food containing staphylococcal enterotoxins. These are produced by enterotoxigenic strains of staphylococci (mainly *S. aureus*) (Bianchi, et al., 2014). Enterotoxin-encoding genes in *S. aureus* isolates were detected in samples of milk, cheese, whey, and swabs from buckets, sieves, and cheese handlers' hands, demonstrating that the consumption of unpasteurized cheese represents a potential public health risk, and underscoring the need for closer surveillance and inspection of the production process of artisanal unpasteurized cheese.

The growth of *S. aureus* and enterotoxins in dairy products can be avoided during food processing by heat treating milk, using starter cultures, controlling the concentration of salt, reducing the pH, and processing and storing cheese at low temperatures. *S. aureus* enterotoxins, however, are much more resistant to environmental factors and food processing than staphylococcal bacterial cells. Although the bacteria are eliminated, the toxins will remain and can cause staphylococcal food poisoning (SCVPH 2000; Hennekinne et al. 2011; Schelin et al. 2011). The use of good hygiene practices in milking is therefore essential to avoid contamination and prevent risks to public health.

In addition, due to the detection of these potentially pathogenic microorganisms, we suggest performing other studies to investigate other microorganisms (*Escherichia coli, Campylobacter spp., Streptococcus* spp., *Brucella abortus*, *Mycobacterium bovis*, *Coxiella burnetti* and *Salmonella enterica*) in artisanal unpasteurized cheeses. Also, research focused on establishing relations among virulence genes in isolates from milk and utensils with cheese produced on farms would be interesting.

These researches are useful for a complete understanding regarding risks to public health due to dairy consumption. In conclusion, *Listeria* spp. and potentially pathogenic *Staphylococcus aureus* was detected in the cheesemaking process in a specific region of the northeastern part of the state of São Paulo, Brazil. This may represent a risk to public health since unpasteurized cheese is widely consumed in this area. So, these findings show that poor hygienic practice is associated with a higher risk of pathogenic bacteria in milk or cheese, providing useful information for public health authorities to increase food safety surveillance and prevent the dissemination of *Listeria* spp. and *Staphylococcus aureus.*

Conclusions

S. aureus carrying virulence genes were detected in milk, swabs from cheese handler's hands, whey, milk, sieves, buckets, and cheese. These findings show that poor hygienic practice is associated with a higher risk of pathogenic bacteria in milk or cheese, providing useful information for public health authorities to increase food safety surveillance and prevent the dissemination of pathogens.

Acknowledgments

The authors gratefully acknowledge the Brazilian research financing agencies CAPES (Federal Agency for the Support and Improvement of Higher Education) and FAPESP (São Paulo Research Foundation, Process no. 2014/13567-1) for their financial support of this work.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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