Microbiological quality of Brazilian artisanal cheese and fermented sausages

Qualidade microbiológica de queijos e salames artesanais brasileiros

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ABSTRACT
Cheeses and sausages are ready-to-eat foods, and the products prepared by artisanal process are susceptible to microbial contamination. Few studies on the quality of foods from different Brazilian regions have been done. In this context, this study aimed at evaluating the microbiological quality of 32 cheese and 13 sausages samples purchased from artisanal food stores or producers markets located in the metropolitan areas of 10 capital cities of Brazilian states. Microbiological analyses were performed to determine the counts of microbial contamination indicators, including Escherichia coli and coagulase-positive staphylococci, as well as for evaluating the presence of Listeria monocytogenes and Salmonella spp. using bacteriological methods. E. coli was detected in 50.0 % of samples, coagulase-positive staphylococci in 34.4 %, and Salmonella spp. in 6.3 % of cheese samples. In fermented sausage samples, coagulase-positive staphylococci were isolated from 23.1 % samples and Salmonella spp. from 7.7 %. According to the Brazilian Sanitary Legislation, 63.0 % of cheese samples and 23.0 % of artisanal fermented sausage samples from the metropolitan areas of 10 Brazilian capital cities were unsuitable for consumption, indicating the importance of conducting the close monitoring of these foods and the application of the effective measures for preventing food-borne outbreaks.

Keywords. Escherichia coli, coagulase-positive Staphylococcus, Listeria monocytogenes, Salmonella, food microbiology.

RESUMO
Queijos e salames são alimentos prontos para consumo e quando artesanalmente produzidos são suscetíveis à contaminação microbiana. A importância deste estudo em dez capitais brasileiras deve-se à carência de dados obtidos simultaneamente abrangendo-se diferentes regiões do Brasil. O objetivo do presente trabalho foi de avaliar a qualidade microbiológica de 32 amostras de queijos e 13 de salames artesanais, adquiridos em casas de produtos artesanais ou feiras de produtores nas regiões metropolitanas de dez capitais brasileiras. Análises microbiológicas com as respectivas contagens de indicadores de contaminação microbiana, Escherichia coli e Staphylococcus coagulase positiva, e a pesquisa de Listeria monocytogenes e Salmonella spp. foram realizadas por meio de métodos bacteriológicos. Nas amostras de queijos foram observadas E. coli em 50,0 %, Staphylococcus coagulase positiva em 34,4 % e Salmonella spp. em 6,3 %. Nas amostras de salames foram detectadas Staphylococcus coagulase positiva em 23,1 % e Salmonella spp. em 7,7 %. De acordo com a legislação sanitária brasileira, 63,0 % das amostras de queijos e 23,0 % das amostras de salames artesanais coletadas na Região Metropolitana de dez capitais brasileiras estavam impróprias para o consumo, o que demonstra a importância de realizar monitoramento próximo e efetivo para prevenir surtos de origem alimentar.

INTRODUÇÃO

Artisanal cheeses are produced in many countries and they have specific properties depending on the region where they are made. Artisanal foods are prepared mainly by family labor to improve their income. In most of the cases, these families do not have sufficient resources to adapt their production facilities to improve the product quality, typically because of the lack of investments from the government agencies responsible for their development. Ready-to-eat (RTE) foods such as cheeses and fermented sausages are in a higher risk category than other foods prepared by using heating steps for cooking. “Homemade” cheese was the second mostly common type reported among those outbreaks caused by cheeses made from unpasteurized milk, occurred from 1998 to 2011.

The occurrence of food-borne diseases has been arising worldwide. Several factors contribute to the emergence of these diseases, such as the increased exposure of the population to RTE foods. Food-borne disease outbreak has been defined as the occurrence of two or more cases of a similar illness resulting from the ingestion of a common food. From 1998 to 2011, 90 outbreaks to which the cheese was the implicated food were reported to the CDC.

These outbreaks resulted in 1882 illnesses, 230 hospitalizations, and six deaths. In Brazil, the majority of the food-borne diseases have been caused by bacteria such as Salmonella spp., Escherichia coli, Staphylococcus aureus, Shigella spp., Bacillus cereus, and Clostridium perfringens.

In order to evaluate the microbiological quality of the food from animal origins, the quantification of E. coli and the detection of Salmonella are used, because these microorganisms are reliable indicators for evaluating the food quality and safety worldwide. E. coli is a major commensal inhabitant of the intestine of humans and warm-blooded animals, and it does part of the essential microbiota that maintains the physiology of healthy hosts. This bacterium is also considered as a microbiological indicator of fecal contamination or of processing quality; additionally, some E. coli strains can cause diarrhea.

Coliforms are microbial indicators for hygiene in food processing and they might be influenced by factors like the quality of raw materials, failure during processing, or contamination during post-processing phase in pasteurized foods. They are easily destroyed by heat and they usually cannot survive the heat treatment.

The presence of S. aureus in milk and its products suggests the use of raw materials from infected animals (mastitis) or a probable contaminating agent introduced by handlers who are asymptomatic carriers. The causative agents of staphylococcal food poisoning include the staphylococcal enterotoxins (SEs) and the exotoxins pre-formed by S. aureus in food. SEs are produced when the pathogen population is higher than or equal to 5 log CFU.g⁻¹ (Colony Forming Unit) of food.

Salmonellosis is a zoonosis of great importance and a major public health concern worldwide. The most prevalent serovars of Salmonella spp. vary geographically and over time. Overall, in the USA, the salmonellosis incidence has not decreased over the past several decades; the incidence has substantially increased for some serotypes and decreased for others.

Food processors, particularly those producing RTE products, should be vigilant to Listeria monocytogenes, the causative pathogen of listeriosis. Listeria monocytogenes is ubiquitous in the environment and it can survive for long periods of time in seemingly inhospitable environments such as the food processing facilities because of its ability to resist various stresses and to form biofilms. L. monocytogenes is capable to replicate at refrigeration temperatures, and thus it is of a particular concern in products with a long shelf-life. From the public health perspective, L. monocytogenes shows the strongest impact in immunocompromised individuals, and it is a food-borne pathogen with the highest mortality rate (28.0%). No compulsory notification
for listeriosis cases is needed in Brazil, but in order to monitor and to ensure the safety of RTE products from animal origin, the Brazilian Ministry of Agriculture, Cattle Raising, and Supply\textsuperscript{11} established the criteria and procedures for \textit{L. monocytogenes} control. The Brazilian National Health Surveillance Agency also establishes the absence of this pathogen in 25 g of cheese sample\textsuperscript{11,15}.

The present investigation is crucial as the few studies have been conducted simultaneously and covering different regions of Brazil. The metropolitan areas were included in the analysis mostly because some municipal health monitoring services are effective for the artisanal foods sale.

ISO methods for microbial analysis were performed for carrying out the earlier isolation of characteristic colonies onto the surface of the primary agar plates. On the contrary when the conventional methods as the multiple tube technique is used, it takes longer. In general, the chromogenic media are able to isolate and to distinguish the bacteria species from the others, and it allows a more rapid detection, for example, and distinguishing \textit{L. monocytogenes} from other \textit{Listeria} sp\textsuperscript{16}.

Fermented sausages and cheese samples were analyzed because of their animal origin, and they are artisanal RTE foods, which do not require any thermal treatment before consumption, and the probability of isolating potential pathogenic microorganisms from them is high.

The aim of this study was to determine the microbial quality of the Brazilian RTE artisanal foods such as cheeses and fermented sausages, which are commercially available at artisanal food houses or at producer fairs in the metropolitan areas of the capital cities of ten Brazilian States. Microbiological indicators such as the hygienic sanitary quality of raw material and the processes or detection of pathogenic bacteria, such as coliforms, \textit{E. coli} and coagulase-positive staphylococci were evaluated, and the occurrence of pathogenic bacteria \textit{Salmonella} spp. and \textit{L. monocytogenes} was determined.

**MATERIAL AND METHODS**

**Sample collection and identification**

A total of 45 samples of RTE artisanal foods, including 32 cheese samples and 13 fermented sausage samples, were purchased from February to May 2013 at artisanal food houses or producer fairs in the metropolitan areas of the capital cities of the ten States of Brazil. The number of samples from each city was as follows (cheese/fermented sausage): Porto Alegre (3/3), Curitiba (4/3), São Paulo (3/3), Belo Horizonte (4/1), Salvador (3/0), Recife (3/0), Natal (3/0), Fortaleza (3/3), Manaus (3/0), and Brasília (3/0). The samples were obtained from different producers, and none of them had the sanitary inspection seal.

The microbial quantification results are expressed as log CFU.g\textsuperscript{-1}, and the presence of pathogens per 25 g is indicated as 1 log.

**Microbiological analysis**

Microbial quantification of indicators, including \textit{E. coli} and coagulase-positive staphylococci, and microbial studies of pathogenic bacteria \textit{L. monocytogenes} and \textit{Salmonella} spp. were performed as described below. Coliforms were quantified in order to complement the data of contamination levels using microbial indicators of hygiene\textsuperscript{7}.

To quantify coliforms, \textit{E. coli}, and coagulase-positive staphylococci, the aliquots of 25 g of cheese or fermented sausages samples were homogenized with 225 mL of sterile water containing 0.1 % peptone, and decimal dilutions were prepared using the same diluent\textsuperscript{17} (Laborclin, Pinhais, Brazil).

\textit{E. coli} and coliforms were determined by following the ISO 4832\textsuperscript{18} and ISO 16649-2\textsuperscript{19} methods on a Compass\textsuperscript{®} ECC chromogenic agar (Biokar Diagnostics, Paris, France) surface, followed by incubation at 35 ± 1 °C for 24 ± 2 h\textsuperscript{18}. This medium included two chromogenic substrates, rose-galactoside and X-glucuronide, for simultaneously detecting coliforms and \textit{E. coli}. The rose colonies were considered to indicate the occurrence of coliforms possessing \(\beta\)-galactosidase activity and the dark blue colonies as \textit{E. coli} possessing \(\beta\)-galactosidase and...
β-glucuronidase activities\textsuperscript{19,20}.

Three colonies suspected to contain \textit{E. coli} for each sample were confirmed by purification on Cromoclin US agar (Laborclin) and they were phenotypically identified using Bactray\textsuperscript{®} 1 and 2 systems (Laborclin).

The microbiological samples processing for isolating coagulase-positive staphylococci was conducted using the ISO 6888-1 method\textsuperscript{21} with Baird Parker agar (Laborclin) followed by incubation at 35 ± 1 °C for 24 – 48 h. Strains of coagulase-positive staphylococci were identified upon observation of catalase- and coagulase-positive Gram-positive cocci.

\textit{Listeria} was detected following the ISO BS EN ISO 11290-1 method, which included homogenization of 25 g of the sample with 225 mL Demi Fraser broth, (BD Diagnostics, Sparks, USA). The enrichment broth was incubated at 30 ± 1 °C for 24 h. Primary Fraser broth cultures were transferred to secondary Fraser broth (Laborclin) and streaked onto Palcam agar (Laborclin) and ALOA (Agar \textit{Listeria} according to Ottaviani and Agosti) agar (Laborclin). Fraser broth and agar plates were incubated at 35 ± 1 °C for 48 ± 2 h. Secondary Fraser broth was streaked on Palcam agar and ALOA agar plates and incubated at 35 ± 1 °C for 48 ± 2 h. All of the plates were analyzed for detecting the occurrence of \textit{Listeria} colonies, which appeared as small dark brown colonies on Palcam agar, and small blue colonies on ALOA agar, with or without a surrounding opaque zone. The purified isolates, which included those that were blue on Cromoclin US agar, the Gram-positive coccobacilli showed positive catalase activity with 3 % hydrogen peroxide. Those presenting in the motility test at 25 °C with a typical umbrella shape in SIM medium (Laborclin), they were identified as \textit{Listeria} spp. Carbohydrate fermentation tests were conducted in purple carbohydrate broth (Laborclin) and Rapapport-Vassiliadis Soy (RVS) broth (Laborclin), and incubating at 37 ± 1 °C and 41.5 ± 1 °C for 24 h, respectively. Enrichment cultures were streaked onto XLD agar (Laborclin) and chromogenic \textit{Salmonella} agar (Laborclin). The plates were incubated at 37 ± 1 °C for 24 ± 2 h. Purified isolates, which were white on Cromoclin US agar, were identified using the miniaturized system Bactray\textsuperscript{®} (Laborclin). Serological assays such as polyvalent flagellar (H) and polyvalent somatic (O) tests were performed by using anti-\textit{Salmonella} agglutinating sera (Probac, São Paulo, Brazil).

**Colony purification**

Although the ISO methods suggest to use nutrient agar for colony purification, the suspected colonies on primary media were inoculated on the surface of Cromoclin US agar (Laborclin) before performing the phenotypic identification, because this nutrient medium was added with chromogenic substrate to facilitate the visualization of characteristic colonies. On this chromogenic media, coliforms appear as dark blue colonies owing to X-glucoside and rose-galactoside consumption, \textit{E. coli} consumes rose-glucuronide and rose-galactoside, where as \textit{Enterococcus} spp. and \textit{Listeria} spp. consume X-glucoside, and their color is light blue. Rose colonies in the Baird Parker agar were discharged because of the galactosidase activity of \textit{Staphylococcus}. Coagulase-positive staphylococci and \textit{Salmonella} spp. cannot consume these substrates and they appear as white to yellowish colonies. In the present study, this medium enabled to distinguish \textit{Proteus} spp. from XLD agar and chromogenic \textit{Salmonella} agar, which appeared as brown colonies owing to the tryptophanase activity.

**RESULTS**

The populations of coliforms, \textit{E. coli}, coagulase-positive staphylococci, \textit{Salmonella} spp., and \textit{Listeria} spp. isolated from artisanal cheese and fermented sausage samples purchased in the metropolitan areas of ten Brazilian States capitals are reported in Table 1.
Table 1. Populations of coliforms, *E. coli*, coagulase-positive staphylococci, *Salmonella* spp. and *Listeria* spp. from artisanal cheese and fermented sausages samples bought in metropolitan areas of ten Brazilian cities

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<th>E. coli (log CFU.g⁻¹)</th>
<th>Coagulase-positive staphylococci (log CFU.g⁻¹)</th>
<th>Salmonella spp.</th>
<th>Listeria spp.</th>
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a. Improper sample: Containing *E. coli*, coagulase-positive staphylococci, and/or *Salmonella* spp. above the counts permitted by regulation; b. Reference values in accordance to the Brazilian Health Regulatory Laws; c. Cheese with very high humidity (>55 %); d. Considered data from coliforms at 45 °C.
Among the 45 samples acquired from the artisanal food houses or producer fairs in the metropolitan areas of ten Brazilian States capital cities, 62.5% of artisanal cheeses samples (20/32) and 23.1% of artisanal fermented sausage samples (3/13) were inadequate for human consumption. In accordance with the Brazilian Health Regulatory Legislation\textsuperscript{15}, 51.1% of the samples (23/45) were not suitable for consumption due to the presence of \textit{E. coli} and/or coagulase-positive staphylococci and/or \textit{Salmonella} spp. at levels higher than those allowed by legislation.

The coagulase-positive staphylococci counting was higher than the maximum allowed values of 3.7 log CFU.g\textsuperscript{-1} and 2.7 log CFU.g\textsuperscript{-1} in 23.1% for the fermented sausage samples (3/13) and in 34.4% cheese samples (11/32), respectively, at very high humidity (>55.0%); and these values were not in agreement with the Brazilian Regulatory Legislation\textsuperscript{15}.

Coliforms were isolated from 81.25% of the cheese samples (26/32) with counts of 3.0–8.5 log CFU.g\textsuperscript{-1} and from 23.1% of fermented sausage samples (3/13) with 4.3–4.9 log CFU.g\textsuperscript{-1} counting.

Although \textit{E. coli} was not isolated from fermented sausages samples, 56.3% of cheese samples (18/32) showed values between 2.4 and 6.3 log CFU.g\textsuperscript{-1}. Considering that \textit{E. coli} belongs to the coliform group at 45 °C\textsuperscript{6}, and being 2.7 log CFU.g\textsuperscript{-1} the maximum allowed value for the very high humidity cheeses\textsuperscript{15}, 50.0% of cheese samples (16/32) were improper for consumption.

\textit{Salmonella} spp. was isolated from 6.3% of cheese samples (2/32) and 7.7% of fermented sausages samples (1/13).

\textit{Listeria} spp. was isolated from 6.3% of cheese samples (2/32) and from 30.8% of fermented sausages samples (4/13); however, the presence of \textit{L. monocytogenes} was not confirmed.

**DISCUSSION**

\textit{E. coli}, coagulase-positive staphylococci, and/or \textit{Salmonella} spp. were found in the analyzed samples, corroborating the results reported by Almeida et al\textsuperscript{24}. These investigators found that these microorganisms were among the most common etiological agents detected in the positive samples from patients during the food-borne disease outbreaks occurred in Parana state, Brazil.

In the present study, the found results for coagulase-positive staphylococci were comparable to those of previous studies performed by Evêncio-Luz et al\textsuperscript{25}, who reported the occurrence of these bacteria in Salvador and Recife. A total of 21.0% (16/75) and 31.2% (20/65) of \textit{coalho} type cheese samples were improper for human consumption.

Among 30 samples, Visotto et al\textsuperscript{7} found four samples (13.3%), being two industrialized and two artisanal varieties were contaminated with coagulase-positive staphylococci above 5 log CFU.g\textsuperscript{-1}. The results detected in the present study are in accordance with the findings reported by those authors\textsuperscript{7}, as among 32 cheese analyzed samples, four (12.5%) contained coagulase-positive staphylococci above 5 log CFU.g\textsuperscript{-1}. Borelli et al\textsuperscript{26} found \textit{Staphylococcus} spp. at levels higher than 5.0 log CFU.g\textsuperscript{-1} in all of the analyzed cheese samples from the Serra da Canastra region, Minas Gerais State, and among them 93.3% were capable to produce the staphylococcal enterotoxins.

For coliforms, this study found similar results to those detected in cheese by Visotto et al\textsuperscript{7}, showing that 90.0% of the analyzed samples (27/30) contained populations higher than 3.0 log MPN.g\textsuperscript{-1} (Most Probable Number). The authors stated that the high occurrence of these microbial indicators suggested that the cheeses were produced with poor-quality raw materials or that failures had occurred during the manufacture and storage processes.

In a previous study, the evaluated cheese samples were deemed improper for consumption because of \textit{E. coli} contamination as reported by Visotto et al\textsuperscript{7}. These authors observed that 50.0% of artisanal cheese samples (4/8) and 54.5% of industrial cheese samples (12/22) purchased in Ribeirão Preto, São Paulo, Brazil contained thermotolerant coliform populations at levels above those permitted by the Regulations. Borelli et al\textsuperscript{26} found high levels of thermotolerant coliforms in cheese samples from Serra da
Canastra region, Minas Gerais state and reported the presence of this microorganism in all of the water samples used for cheese production.

In contrast to the results reported by Osailli et al\textsuperscript{27} who isolated *Listeria* spp. from 27.1 % cheese samples and *L. monocytogenes* from 39 (11.1 %) Brined White Cheese samples in Jordan, the present study isolated *Listeria* spp. but not *L. monocytogenes*.

*Salmonella* spp. and *Listeria* spp. isolated in the present study raises the concerns regarding the risks to human health. Gould et al\textsuperscript{2} reported outbreaks attributed to cheese in the USA from 1998 to 2011, wherein three deaths were associated with cheeses made from unpasteurized milk contaminated with *Listeria* (two fatal cases) and with *Salmonella* serotype Typhimurium. Three deaths occurred in outbreaks resulting from cheeses made from *Listeria* contaminated-pasteurized milk. Kottwitz et al\textsuperscript{8} reported that meat derivatives and cheese were among the foods associated with human salmonellosis outbreaks in Parana between 1999 and 2008, wherein a total of 286 outbreaks were analyzed; and 14.0 % of outbreaks were associated with meat derivatives and 1 % with cheese.

The low occurrence of *Salmonella* spp. and the absence of *L. monocytogenes* in cheese samples evaluated in the present study could be explained based upon the findings reported by Visotto et al\textsuperscript{7} and Silva et al\textsuperscript{28}. These authors presented data showing that *Salmonella* spp. and *L. monocytogenes* might be absent in cheese samples due to the occurrence of autochthonous microbiota. This microbiota might constrain the growth of pathogenic microorganisms by lowering the competitive ability of such species over other bacteria occurring in the cheeses and/or by producing antagonistic molecules.

In the fermented sausages evaluated in the present study, the absence of *L. monocytogenes* and *E. coli* counts in the fermented sausage. Porto-Fett et al\textsuperscript{30} reported that fermentation in pH 4.8 and storage at 21 °C were effective for reducing the numbers of *L. monocytogenes* (2.54 log CFU.g\textsuperscript{-1} reduction) and *Salmonella* serotype Typhimurium (≥ 5.23 log CFU.g\textsuperscript{-1} reduction).

The results obtained in different regions suggest that the northeast region includes the largest number of samples improper for consumption, wherein 75.0 % of analyzed cheese samples (9/12) were noncompliant with the Brazilian Health Regulatory Legislation\textsuperscript{15}. Only a few Brazilian states and/or municipalities have compiled statistics and data regarding the most common etiologic agents, being the foods commonly involved in outbreaks, the population at high risk, and the factors contributing to food-borne diseases\textsuperscript{3}. To improve the quality of artisanal Minas cheese, the government of Minas Gerais has instituted the State Regulatory Standards for establishing the physicochemical and microbiological parameters to which these cheeses must comply with\textsuperscript{1}.

**CONCLUSION**

The high occurrence of microorganisms detected in the microbiological analyses performed in some of the ten states capital cities indicated the low hygienic sanitary quality of the produced cheese samples. The occurrence of pathogens in some samples represents a potential risk to consumer health, emphasizing the importance of the close and effective monitoring by the Health Surveillance service to prevent food-borne outbreaks. The rapid detection of pathogenic bacteria in food is crucial and it is fundamental for preventing the food-borne illness outbreaks.

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