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FOOD PRODUCTION

Microbiological quality and safety assessment in the production of moderate and high humidity cheeses

Denise da Fontoura Prates[1] Simone Rauber Würfel[1] Júlia Coswig Goldbeck[1] Andreia Saldanha de Lima[1] Graciela Volz Lopes[1] Wladimir Padilha da Silva[1*]

[1]Departamento de Ciência e Tecnologia de Alimentos, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas (UFPel), Pelotas, RS, Brasil. Email: silvawp@ufpel.edu.br. *Corresponding author.

ABSTRACT: *Moderate and high humidity cheeses are described as important vehicles of pathogens in many foodborne diseases outbreaks. Microbial contamination can occur in raw material or in the different steps of the product processing due to inadequate hygiene practices. Thus, the aim of this study was to evaluate the microbiological quality and safety in the production of moderate and high humidity cheese. Samples from raw milk, handlers' hands surface, final product were collected in three cheese manufacturing plants located in southern Brazil, with different levels of sanitary control. Effectiveness of milk pasteurization was also evaluated. Thermotolerant coliforms, coagulase-positive staphylococci (CPS), Salmonella spp., and Listeria monocytogenes were evaluated. Raw milk samples showed the highest contamination levels, with enumeration of $1.1x10^5$ most probable number (MPN) mL^{-1} for thermotolerant coliforms, $4x10^5$ colony-forming units (CFU) mL^{-1} for CPS and presence of Salmonella spp. CPS were also reported in one sample of handler's hands surface. However, only one sample of the final product was out of Brazilian regulatory standards, exceeding the limit allowed for CPS. Milk pasteurization process used in cheese preparation was effective, regardless the level of sanitary control of the industries. Results highlighted the need for better hygiene practices, in obtaining the raw milk and in the handling during the cheese manufacturing steps.*
Key words: *Listeria monocytogenes, Salmonella spp., thermotolerant coliforms, coagulase-positive staphylococci, food safety.*

Qualidade microbiológica e avaliação da segurança na produção de queijos de média e alta umidade

RESUMO: *Os queijos com média e alta umidade são alimentos prontos para o consumo, que têm sido descritos como veiculadores de patógenos em diversos surtos de doenças transmitidas por alimentos. A contaminação microbiana pode ter origem na matéria prima, ou ocorrer durante as etapas de elaboração do produto, através de práticas inadequadas de higiene. Dessa forma, o objetivo deste estudo foi avaliar a qualidade e a segurança microbiológica na produção de queijos de média umidade. Amostras da matéria prima, dos manipuladores e do produto final foram coletadas em três laticínios situados na região sul do Rio Grande do Sul, com diferentes níveis de inspeção sanitária. A eficiência da pasteurização do leite também foi avaliada. Coliformes termotolerantes, estafilococos coagulase-positivos (ECP), Salmonella spp. e Listeria monocytogenes foram avaliados. As amostras de leite cru foram as que apresentaram os maiores níveis de contaminação, com enumeração de $1,1x10^5$ número mais provável (NMP) mL^{-1} para coliformes termotolerantes, $4x10^5$ unidades formadoras de colônia (UFC) mL^{-1} para ECP e a presença de Salmonella spp.. Contudo, apenas uma amostra de produto final estava em desacordo com o padrão regulamentar vigente, excedendo o limite permitido para ECP. A pasteurização do leite utilizado no preparo dos queijos foi eficiente em todos os laticínios, independentemente do nível de inspeção sanitária dos estabelecimentos. No entanto, houve contaminação pré e pós-pasteurização, demonstrando a necessidade de melhores práticas higiênicas, tanto na obtenção da matéria-prima, quanto na manipulação durante as diversas etapas de fabricação dos queijos.*
Palavras-chave: *Listeria monocytogenes, Salmonella spp., coliformes termotolerantes, estafilococos coagulase positiva, segurança alimentar.*

INTRODUCTION

The presence of pathogens in milk products are a major concern in public health (SOBRINHO et al., 2012) and cheese consumption has been associated with foodborne disease outbreaks (FBDOs) in different parts of the world, since natural cheeses can support the growth of microorganisms including foodborne pathogens (CHOI et al., 2016). According to Brazilian regulatory standards, use of raw milk in ripened cheese production for a period less than 60 days is only allowed when technical and scientific studies attested that reducing the period will not compromise the product quality and safety (BRAZIL,

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2013). Pasteurization is the safest method to destroy bacterial pathogens commonly reported in raw milk. Nevertheless, contamination of cheese with foodborne pathogens can occur during the several manufacture steps and from various sources (KOUSTA et al., 2010).

Listeria monocytogenes, *Salmonella* spp. and *Staphylococcus aureus* are pathogenic bacteria often associated to FBDOs due to contaminated cheese consumption, are ubiquitous and can be isolated from raw milk, equipments, utensils and handlers' hands (KOUSTA et al., 2010). Investigation and control of microbial contamination in the processing line are relevant for food industry, because presence of pathogens in the final products generates economic losses due to recall of products exceeding legal standards, which compromises the image of industry and jeopardises public health (EFSA/ECDC, 2014).

Prato cheese is one of the most popular cheeses in Brazil, and it is an enzymatically coagulated cow's milk cheese, ripened, semi-hard, with a mild flavor, pale yellow color, and moderate humidity (36%-45.9%) (BRAZIL, 1997). Coalho cheese is a soft cheese obtained after milk coagulation using curdle or proper coagulating-enzymes, sometimes complemented with lactic bacteria. It is characterized by a mild acidic flavor, pale yellow color, and high humidity (46%-54.9%) (BRAZIL, 2001a). In Brazil, there are microbiology requirements for moderate and high humidity cheeses. The parameters are absence of *L. monocytogenes* and *Salmonella* spp. in 25g of sample, and maximum count of 10^3 colony-forming units (CFU) g^{-1} for coagulase-positive staphylococci (CPS). Regarding thermotolerant coliforms (coliforms at 45ºC), the maximum parameters allowed are 10^3 and $5x10^3$ most probable number (MPN) g^{-1} for moderate and high humidity cheeses, respectively (BRAZIL, 2001b). Therefore, the aim of this study was to evaluate the microbiological quality and safety in the production of moderate and high humidity cheese.

MATERIAL AND METHODS

Sampling procedure

Three cheese manufacturing plants from southern Brazil were evaluated. In dairy plant A (small industry with official sanitary control in implementation phase), the manufacturing of Coalho cheese (high humidity; 46% < hum < 54.9%) was evaluated. In both dairy plant B (small industry, inspected by Rio Grande do Sul State – DIPOA/SEAPI) and dairy plant C (medium-size industry, inspected by Brazilian Federal Government – SIF), manufacturing of Prato cheese (moderate humidity; 36% < hum < 45.9%) was evaluated. Dairies were selected due to produce moderate and high humidity cheeses (commonly involved in FBDOs) and with different levels of sanitary control.

A total of 24 samples were collected from raw milk (n=6), pasteurized milk (n=6), handlers' hands surface (n=6) and packaged cheese (n=6) at dairy plants A, B and C. Sampling was conducted in accordance with methodology recommended by American Public Health Association (APHA, 2001), with adaptations. During each sampling procedure, raw and pasteurized milk were collected in sterilized bottles. Handlers' hands that worked during the manufacturing process of products were sampled using the modified superficial washing technique (SILVA et al., 2007) in bags containing 100mL of saline solution (0.85%). Final product samples were obtained the next day in their commercial packaging. The samples were refrigerated and immediately transported to the laboratory in isothermal containers with ice for microbiological analysis.

Thermotolerant coliforms enumeration, CPS count and *Salmonella* spp. presence were performed in all samples collected. *Listeria monocytogenes* was verified only in the cheese samples. Analytical units of 25mL or 25g from each sample were used.

Thermotolerant coliforms enumeration

To each sample, 225mL of buffered peptone water 0.1% (Acumedia, Lansing, USA) was added. The analyses were performed using the multiple tube test (APHA, 2001). Briefly, samples were diluted and aliquots were transferred to test tubes containing Lauryl Tryptose broth (Oxoid, Basingstoke, UK), incubated at 35°C for 24-48h. Test tubes that showed positive results were aliquoted to test tubes containing EC broth (Oxoid) and Brilliant Green Bile Lactose broth 2% (BGB) (Oxoid), which were incubated at 45°C for 24-48h. For milk samples, aliquots of the diluted samples were transferred directly to EC and BGB broth. Densities of thermotolerant coliforms in the samples were obtained from the most probable number (MPN) table (APHA, 2001).

Coagulase-positive staphylococci count

To each sample, 225mL of buffered peptone water 0.1% (Acumedia) was added. According to APHA (2001), for CPS analysis, decimal dilutions of samples were inoculated on Baird Parker agar (Acumedia) and incubated at 35°C for 48h. After incubation, a presumptive count of colony-forming units (CFU) was performed, the presumptive colonies

were selected and transferred to Brain Heart Infusion broth (Difco, Sparks, USA), and confirmation was performed by phenotypic tests of Gram staining, catalase and coagulase production.

Salmonella spp.

To each sample, 225mL of buffered peptone water 0.1% (Acumedia) was added. For selective enrichment, aliquots were transferred to test tubes containing Tetrathionate (Oxoid) and Rappaport Vassiliadis (Oxoid) broths, and the respective tubes incubated at 37°C for 24h and at 42°C for 24h. Thereafter, two selective media were used: Hektoen Enteric (Oxoid) and Xylose Lysine Deoxycholate (Oxoid) agars, incubated at 37°C for 24h. Colonies with typical characteristics of *Salmonella* spp. were subjected to phenotypic tests in Triple Sugar Iron (Acumedia), Lysine iron (Acumedia), and Urea (Acumedia) agars, incubated at 37°C for 24h. Somatic and flagellar antisera (Probac, São Paulo, Brazil) were used to serologic confirmation (APHA, 2001).

Listeria monocytogenes

To each sample, 225mL of Listeria Enrichment broth (LEB-Oxoid) was added. According to methodology described (FARBER et al., 1994), Fraser broth (Oxoid) was used for selective enrichment and incubated at 37°C for 48h. Later, inoculation was carried out onto Oxford (Oxoid) and Palcam (Oxoid) agars, which are incubated at 37°C for 48h. Typical *Listeria* colonies were subjected to phenotypic identification based on Gram staining, catalase production, motility at 25°C, β-hemolysis production, and fermentation of carbohydrates.

Pasteurization process effectiveness

Pasteurization effectiveness process was verified through the evaluation in the activity of phosphatase and peroxidase enzymes, according to Brazilian regulatory standards (BRAZIL, 2006).

RESULTS AND DISCUSSION

Results of thermotolerant coliforms and CPS enumeration, as well as the occurrence of *Salmonella* spp. in raw and pasteurized milk samples and handlers' hands surface are showed in the table 1. Four samples of handlers' hand surface were contaminated with thermotolerant coliforms, but in low levels. Conversely, all raw milk samples presented thermotolerant coliforms enumeration above 10^3 MPN mL^{-1}. However, there was no detection of this group of microorganisms in pasteurized milk samples (<0.3MPN mL^{-1}), showing the pasteurization effectiveness process used by the dairies evaluated, which was confirmed by results of phosphatase (negative) and peroxidase (positive) tests. High levels of thermotolerant coliforms reported in raw milk samples analyzed can be related to deficient sanitary conditions of dairy farms or to hygienic-sanitary problems in the milking process (MORAES et al., 2009; SOBRINHO et al., 2012; GALINARI et al., 2014; PIERI et al., 2014). Bacteria belonging to group of thermotolerant coliforms colonize mammals' intestine and their detection can indicate the presence of other pathogenic microorganisms of enteric origin. Therefore, they are often used as indicators of fecal contamination (direct or indirect) and of potential risk of zoonotic pathogens in foods (FRANCO & LANDGRAF, 2002).

Pasteurization effectiveness was also evidenced by analysis of *Salmonella* spp., which was detected in one sample of raw milk from dairy plant B, but it was not reported in any pasteurized milk samples. Raw milk contamination with pathogenic bacteria such as *Salmonella* spp., reported in our

Table 1 - Enumeration of thermotolerant coliforms, coagulase positive staphylococci, and detection of *Salmonella* spp. in raw milk, pasteurized milk, and in handlers' hand surfaces, in three dairies (A, B and C) from southern Brazil.

Dairy plant (sampling event)	----------Raw Milk (n=6)----------			----------Pasteurized milk (n=6)----------			------Handlers'hand surface (n=6)-----		
	TC (MPN mL^{-1})	CPS (CFU mL^{-1})	*Salmonella*	TC (MPN mL^{-1})	CPS (CFU mL^{-1})	*Salmonella*	TC (MPN mL^{-1})	CPS (CFU mL^{-1})	*Salmonella*
A (1st)	$1.1x10^5$	-	-	<0.3	-	-	<0.3	$2.4x10^2$	-
A (2nd)	$1.1x10^5$	-	-	<0.3	-	-	0.3	-	-
B (1st)	$1.1x10^4$	$1.5x10^3$	-	<0.3	-	-	0.36	-	-
B (2nd)	$1.1x10^4$	$6.0x10^4$	+	<0.3	-	-	0.15	-	-
C (1st)	$1.1x10^5$	$3.4x10^4$	-	<0.3	-	-	0.36	-	-
C (2nd)	$2.8x10^3$	$4.0x10^5$		<0.3			<0.3		

TC: Thermotolerant coliforms; CPS: Coagulase-positive staphylococci; -: absence; +: presence.

study, is commonly reported, and their occurrence is mainly associated with inefficient hygienic practices, since *Salmonella* spp. is also found in faeces of dairy cows. Thus, good practices are essential to avoid raw milk contamination at farms (KOUSTA et al., 2010).

CPS were isolated from 66.7% (4/6) of raw milk samples and their counting varied between $1.5x10^3$ and $4x10^5$CFU mL^{-1}. One sample of raw milk from dairy plant C presented CPS counts above 10^5CFU mL^{-1}. However, these microorganisms were not reported in pasteurized milk samples. Coagulase-positive *Staphylococcus aureus* is one of the most common pathogens infecting dairy cows and a major causative agent of mastitis. This microorganism is commonly isolated from raw milk and represents a potential risk for public health due to the possibility of staphylococcal toxin production (SOBRINHO et al., 2012). *S. aureus*, the main species of the CPS group, can produce toxin when it reaches 10^5 to 10^6CFU per gram of food. Therefore, cheese storage at abusive temperatures may enable the multiplication of *S. aureus*, to reach enough cell concentrations to produce staphylococcal toxins. Even if the milk has undergone appropriate thermal treatment, which eliminates the viable cells of *S. aureus*, risk of food intoxication cannot be distinguished due to the thermoresistant characteristic of the staphylococcal enterotoxins (O'BRIEN et al., 2009). In Europe, 346 staphylococcal intoxication outbreaks were reported in 2012, with cheese involved in 20% of them (EFSA/ECDC, 2014).

S. aureus is also an important indicator of food hygiene and quality, since the milk contamination can occur by food handler asymptomatic carriers of these microorganisms in association to inadequate hygiene practices (O'BRIEN et al., 2009; KOUSTA et al., 2010). TONDO et al. (2000) evaluated a dairy plant in Rio Grande do Sul (Brazil) and reported that 35.2% of food handlers were asymptomatic carriers of *S. aureus*. In our study, CPS ($2.4x10^2$CFU mL^{-1}) were found in one sample of handlers' hands surface from dairy plant A.

Six cheese samples were microbiologically evaluated according to Brazilian regulatory standards (BRAZIL, 2001b) and the results are showed in table 2. One Coalho cheese sample collected in the first sampling event to dairy plant A was in disagreement to the current Brazilian parameter, since the CPS count was of $7.4x10^5$ CFU g^{-1}, which is above the maximum limit allowed (10^3 CFU g^{-1}). Furthermore, two samples of Prato cheese collected in the dairy plant C were contaminated for CPS, but within the limits established by current standards. The presence of CPS in cheese from dairy plant A above the maximum limit allowed by current standards indicates inadequate post-pasteurization manipulation, which can be related to contamination of handlers' hands surface. The dairy plant A is the only one without official sanitary control (in implementation phase), reinforcing the importance of sanitary inspection to ensure food safety.

Listeria monocytogenes and *Salmonella* spp. were not detected in the final product. None of the samples exceeded the limits established by current Brazilian regulatory standards for thermotolerant coliforms; although, these microorganisms were detected in all cheese samples. Coliforms, as well as CPS, are thermosensitive bacteria that should not survive thermal treatment when properly performed, allowing them to be used as indicators of flaws in manufacturing process or contamination post-pasteurization due to inadequate hygiene conditions (ROBINSON, 2002). Therefore, results of this study showed that the final product was handled improperly, since the thermotolerant coliforms were not detected in milk after pasteurization.

It was observed the need of option and/or readjustment of Good Manufacturing Practices (GMP) in all dairies evaluated, since there was

Table 2 - Microbiological evaluation of Coalho and Prato cheeses produced in three dairies (A, B and C) from southern Brazil.

Sample (Dairy plant)	Thermotolerant Coliforms (MPN g^{-1})	Coagulase-positive Staphylococci (CFU g^{-1})	*Salmonella* spp.	*L. monocytogenes*
CC 1 (A)	$7.4x10^2$	$7.4x10^5$	-	-
CC 2 (A)	2.3x10	-	-	-
PC 3 (B)	9.3x10	-	-	-
PC 4 (B)	9.3x10	-	-	-
PC 5 (C)	7.4x10	$3.1x10^2$	-	-
PC 6 (C)	7.4x10	$9.2x10^2$	-	-

CC: Coalho cheese; PC: Prato cheese; -: absence; +: presence.

contamination in raw milk, in the handlers' hands surface, as well as in the final product; although, the pasteurization process was effective. These results are in agreement with other authors, who reported the need of GMP implementation in dairies, regardless of industry size, but also emphasized the difficulties found to the implementation of actions that minimize the contamination during the food processing (DIAS et al., 2012; CUSATO et al., 2013).

CONCLUSION

The milk pasteurization used in cheeses' manufacture was effective, regardless of sanitary control level of the dairy plants. However, one sample of the final product from the dairy plant without official sanitary control was in disagreement with the Brazilian regulatory standards, reinforcing the importance of the official service to ensure production of safe foods. There was microbial contamination before and after pasteurization, which indicates the need of improvement in the hygienic practices, both in the acquisition of raw material and in cheeses manipulation in different steps during the manufacturing process due to the risks that represent to public health.

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