

## **AEROMONAS IN PROCESSING LINE OF MINAS FRESCAL AND COLONIAL CHEESES**

### *AEROMONAS NO PROCESSAMENTO DE QUEIJOS TIPOS MINAS FRESCAL E COLONIAL*

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#### **SUMMARY**

The aim of this study is to establish possible contamination points and dissemination forms of the bacteria genus *Aeromonas* during the processing of the Brazilian cheeses Minas Frescal and Colonial. Therefore, different products and production points of the process were analyzed to determine the presence of the microorganism. In Minas Frescal cheese, *Aeromonas* spp. was isolated in raw and pasteurized milk, in the environment and on the handlers' hands. *A. caviae* was the most frequently identified species, but *A. sobria* and *A. schubertii* were also isolated. During the processing of Colonial cheese, the species *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii* and *A. jandaei* were isolated in the water, raw milk, after thermal treatment and curd, as well as on the handlers' hands and utensils. The results showed that the genus *Aeromonas* is disseminated throughout different stages of both cheese processes while the raw milk stands out as the main source of contamination in the industrial and handmade processing.

**KEY-WORDS:** Microbiological quality. Cheese. Public health.

#### **RESUMO**

Com o objetivo de estabelecer, durante o processamento do queijo Minas Frescal e Colonial os possíveis pontos de contaminação e a forma de disseminação de bactérias do gênero *Aeromonas*, foram analisados, quanto à presença do microorganismo, diferentes produtos e pontos do fluxograma de produção. Para o Queijo Minas Frescal, *Aeromonas* spp. foram isoladas no leite cru, leite pasteurizado, ambiente de produção e nas mãos dos manipuladores. *A. caviae* foi a espécie mais frequentemente identificada, sendo também isoladas *A. sobria* e *A. schubertii*. Durante o processamento do queijo Colonial, as espécies *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii* e *A. jandaei* foram isoladas a partir da água, mãos dos manipuladores, utensílios, leite cru e após tratamento térmico e de massa coagulada. Os resultados demonstram que o gênero *Aeromonas* encontra-se disseminado nas diferentes etapas do processamento de queijos, destacando-se o leite cru como principal fonte de contaminação para o processamento industrial e artesanal.

**PALAVRAS-CHAVE:** Qualidade Microbiológica. Queijo. Saúde Pública.

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## INTRODUCTION

The genus *Aeromonas* is distributed worldwide and can be isolated from clinical, environmental and food samples. Currently *Aeromonas* spp., especially *A. hydrophila*, is considered an emerging agent in food-borne illnesses that has received special attention due to its ability to multiply at refrigeration temperatures, and is also important deteriorating agent (DASKALOV, 2006; JANDA & ABBOTT, 2010).

Different studies have shown the presence of species of *Aeromonas* spp. in foods of animal and vegetable origin, and also in chlorinated and non-chlorinated water (ISONHOOD & DRAKE, 2002; AMARAL et al., 2006; ROSSI JR et al., 2006; JANDA & ABBOTT, 2010; MARTINELI et al. 2011). Many of these isolates produce virulence factors such as enterotoxins, cytotoxins, hemolysins and have the ability to adhere and to invade epithelial cells. Thus, contaminated food and water are recognized as important transmission vehicles of these microorganisms and are constantly involved in cases of gastroenteritis or even more serious diseases (KIROV, 1993; PALU et al., 2006). However, despite its importance as an agent of foodborne illness, very little is known about the significance of this bacterium in food, especially on the contamination sources and its spread during production, especially for milk and its derivatives. Thus, until the issues related to pathogenicity and behavior of *Aeromonas* spp. are fully understood, their presence in food can be considered a threat to public health, especially in immune-compromised individuals.

*Aeromonas* spp. are important emerging microorganisms in foodborne diseases. However, there is lack of information regarding their presence in products of animal origin; possible sources of contamination, especially for milk and dairy products; and, their ability to multiply in refrigerated foods, crucial knowledge considering the essential role of dairy products in human feeding habits. Therefore, this study aimed at determining the possible contamination sources and spreading points of bacteria of the genus *Aeromonas*, as well as quantifying the micro-organism in the samples during the processing of Frescal Minas Cheese manufactured in a dairy subjected to permanent hygienic and sanitary control and handmade Colonial cheese, determining the potential risk that the analyzed cheeses may pose to the consumer.

## MATERIAL AND METHODS

Microbiological analyses were performed at the Laboratory of Microbiology of the Food Research Center of the Universidade de Passo Fundo, Passo Fundo, RS, while biochemical characterization was performed at the Department of Preventive Veterinary Medicine and Animal Reproduction, Universidade Estadual Paulista, Jaboticabal, SP. The processing steps of Frescal Minas and Colonial cheeses were carefully analyzed to

determine which ones were more susceptible to contamination by *Aeromonas* species and would allow their occurrence in the final product; or, could act as disseminators of microorganisms in different processing stages. The samples were collected during work days while monitoring the production of cheeses.

Frescal Minas Cheese samples were obtained in a large processing plant subjected to permanent sanitary-hygienic control, located in the state of Rio Grande do Sul. Sampling took place during 12 visits to the industry when a total of 25 samples were collected in each of the following ten points: water supply, hands of handlers before and during the workday, the industry environment, equipment surfaces and utensils that come into direct contact with the cheese, raw milk, pasteurized milk, curd, formed curd and cheese ready for consumption, totaling 250 samples.

The Colonial cheese samples were collected directly at a dairy farm, also located in Rio Grande do Sul, which allocates part of its daily production of milk for making artisanal cheese. A total of only 45 samples were collected and analyzed given the small volume processed. Cheese production was monitored during five visits to the dairy farm while samples were collected at the same sampling points of the industrial cheese production with the exception of formed curd step.

In order to isolate the bacteria of the *Aeromonas* genus, selective enrichment in tryptic soy broth (TSB) supplemented with ampicillin<sup>4</sup> at a concentration of 30 mg per liter was initially carried out (ABEYTA JUNIOR et al., 1990).

After incubation, the selective enrichment cultures were plated on phenol-amide-ampicillin red agar (PALUMBO et al. 1985) and dextrin-ampicillin agar (HAVELAAR & VONK, 1988), which were incubated at 28°C for 24 hours and examined for the presence of large yellow colonies (3-5 mm) surrounded by halo resulting from the hydrolysis of starch or dextrin, characteristics of *Aeromonas*. Up to five colonies were inoculated into tilted tubes containing trypticase-soy agar (TSA) and, confirmed the presence of pure cultures, they were plated on agar triple-sugar-iron (TSI) and subjected to the tests of motility, oxidase, catalase and resistance to the vibrio-static agent O/129 (Popoff, 1984). The characterization of the species was performed following the scheme of Popoff (1984), plus evidence recommended by Abeyta Jr. et al. (1990), and updated by Furuwatari et al. (1994) and Abbott et al. (2003).

*Aeromonas* counts were performed on red agar plates using phenol-amide-ampicillin, plated on the surface in 0.1-mL volumes of each sample or dilution. For confirmation of the genus, up to five characteristic colonies were isolated and submitted to identification evidence.

The nonparametric chi-square test was used to compare the isolation frequency of *Aeromonas* bacteria in industrial and colonial cheese samples (STEEL & TORRIE, 1960).

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<sup>4</sup> Sigma - A9393

## RESULTS AND DISCUSSION

The analysis of the different products at different production stages of the two cheeses, Minas Frescal and Colonial, revealed the presence of *Aeromonas* spp. in 16.6% of samples. For Minas Frescal cheese, which is produced under permanent sanitary-hygiene control, the contamination by the micro-organism was found in the hands of workers during the workday, in the environment, in both raw and pasteurized milk, with 8.0%; 20.0%; 96.0%; and, 1.0% of positive samples, respectively. The species *A. caviae*, *A. sobria* and *A. schubertii* were isolated (Table 1). Furthermore, *A. hydrophila*, *A. caviae*, *A. sobria*, *A. veronii* and *A. jandaei* were identified during Colonial cheese production. With the exception of cheese ready for consumption and the environment, all other points were positive for the bacteria, representing 37.8% of samples (Table 2).

It is noteworthy the fact that raw milk presents the potential risk as the largest disseminator of *Aeromonas* spp. in the dairy industry, as 24 (96.0%) samples were positive for the genus with average population of  $1.0 \times 10^7$  UFC/mL. *A. caviae*, *A. sobria*

and *A. schubertii* were identified among the species isolated. In addition, the result is also alarming for the artisanal cheese production, *A. hydrophila*, *A. caviae* and *A. veronii* were isolated in 60.0% of the samples with a population average of  $2.2 \times 10^4$  UFC/mL.

Several authors have reported the presence of *Aeromonas* in raw milk and its derivatives. Carneiro & Rossi Junior (2006) found the possible *Aeromonas* spp. contamination and spreading points in the processing flowchart of a dairy farm that produced Type A milk in the State of São Paulo and found 90.0% of positive samples for the genus in raw milk. In the same state, Amaral et al. (2006) reported 85.0% and 55.0% of samples positive for *Aeromonas* in the rainy and dry seasons, respectively.

In the case of industrial production, milk storage time and the large distances between rural properties and the dairy farm may have contributed to the high counts obtained. Milk, even under refrigeration, is a medium that favors the spreading of *Aeromonas*. Despite the fact that it is a mesophilic bacteria, studies have shown that the genus is able to multiply at low temperatures, compete with psychrotrophic microbiota, and even produce virulence factors in these conditions (DASKALOV, 2006; JANDA & ABBOTT, 2010).

**Table 1** - Species and populations of *Aeromonas* spp. obtained from samples taken during the production of Frescal Minas cheese, in the period 2006-2008, in northwestern Rio Grande do Sul state.

Origin	Samples			Identified species
	Analyzed	Positive (%)	Counts	
Water	25	0 (0.0)	<1.0x10UFC/mL	-
Hands at the beginning of workday	25	0 (0.0)	<1.0x10UFC/hand	-
Hands at the end of workday	25	02 (8.0)	1.0x10UFC/hand	<i>A. caviae</i>
Utensils	25	0 (0.0)	<1.0x10UFC/cm <sup>2</sup>	-
Environment	25	05 (20.0)	2.1x10 <sup>1</sup> UFC/plate	<i>A. caviae</i>
Raw milk	25	24 (96.0)	1.0x10 <sup>7</sup> UFC/mL	<i>A. caviae</i> , <i>A. sobria</i> , <i>A. schubertii</i>
Pasteurized milk	25	01 (4.0)	1.0x10 UFC/mL	<i>A. sobria</i>
Coagulated milk	25	0 (0.0)	<1.0x10 UFC/g	-
Molded curd	25	0 (0.0)	<1.0x10 UFC/g	-
Cheese ready for consumption	25	0 (0.0)	<1.0x10 UFC/g	-
<b>TOTAL</b>	<b>250</b>	<b>32 (12.8)</b>		

**Table 2** - Species and populations of *Aeromonas* spp. obtained from samples taken during the production of Colonial cheese, in the period 2006-2008, in northwestern Rio Grande do Sul state.

Samples				
Origin	Analyzed	Positive (%)	Counts	Analyzed samples
Water	05	3 (60.0)	7.3x10UFC/mL	<i>A. hydrophila</i> , <i>A. caviae</i>
Hands at the beginning of workday	05	3 (60.0)	4.0x10UFC/mão	<i>A. hydrophila</i> , <i>A. sobria</i>
Hands at the end of workday	05	2 (40.0)	1.0x10UFC/mão	<i>A. veronii</i> , <i>A. jandaei</i>
Utensils	05	1 (20.0)	1.0x10UFC/cm <sup>2</sup>	<i>A. caviae</i> , <i>A. sobria</i>
Environment	05	0 (0.0)	<1.0x10UFC/placa	-
Raw milk	05	3 (60.0)	2.2x10 <sup>4</sup> UFC/mL	<i>A. hydrophila</i> , <i>A. caviae</i> , <i>A. veronii</i>
Heated milk	05	2 (40.0)	1.0x10 <sup>5</sup> UFC/mL	<i>A. hydrophila</i>
Coagulated milk	05	3 (60.0)	6.7x10 <sup>3</sup> UFC/g	<i>A. hydrophila</i> , <i>A. caviae</i> , <i>A. veronii</i>
Cheese ready for consumption	05	0 (0.0)	<1.0x10UFC/g	-
<b>TOTAL</b>	<b>45</b>	<b>17 (37.8%)</b>		

Environmental and water contamination in the rural properties may also have contributed to the high counts observed in raw milk. *Aeromonas* species are widely distributed in the environment, especially in water, soil and animal feces (PALU et al., 2006). Thus, they can easily contaminate the udder of lactating cows and also the equipment used to obtain the raw material through cross-contamination. Water can be an important source of milk contamination even during milking stage. This fact is of particular importance in dairy farms of the study area, which usually use water obtained from deep or shallow wells without chlorination or potability evaluation.

As for the sources of contamination during milk production, the water used on the farm for the production of Minas Frescal cheese was positive for the genus unlike the results observed in the processing of Minas cheese. Of the five water samples, three (60.0%) were contaminated while *A. hydrophila* and *A. caviae* were isolated. This result confirms that *Aeromonas* species are able to multiply in water distribution systems, and untreated water represents an important source of contamination and spreading of *Aeromonas* spp.

We also emphasize the public health risk posed by the supply water used in this property. Burke et al. (1984) reported that *Aeromonas* spp. are an important agent of gastroenteritis and other diseases by isolating the microorganism from patients with diarrhea and chlorinated and non-chlorinated water samples in Australia. The authors found that 91.0% of isolates from diarrhea, 70.0% of isolates from water supply and 64.0% of isolates from non-chlorinated water were enterotoxigenic and haemolytic.

The results presented in this study combined with data from the literature (KIROV et al., 1993; DASKALOV, 2006; JANDA & ABBOTT, 2010) suggest that in the case of Colonial cheese, the water may have been one of the contamination and spreading sources of *Aeromonas* spp. during production. On the other hand, in the industry, this source was eliminated entirely since the genus was not isolated in any of the 25 water samples analyzed.

The hands of the workers were also evaluated. In the production of Frescal Minas cheese, the agent was isolated from the workers hands along the production process. *A. caviae* was isolated in 8.0% of the samples with a count of 1.0 x 10 UFC/hand.

Furthermore, for the Colonial cheese, *A. hydrophila* and *A. sobria* were isolated in 60.0% of samples taken from the workers hands before the start of activities, with a population of  $4.0 \times 10$  UFC/hand, showing that the worker might reach the workplace with the hands already contaminated by such micro-organisms. During the workday, 40.0% of samples from workers hands were positive for *A. veronii* and *A. jandaei*, with average population of  $1.0 \times 10$  UFC/hand. These results show that the workers hands can also represent important contamination source of this pathogen. Therefore, this result shows the importance of good manufacturing practices, especially proper disinfection of the workers hands.

Other authors have also reported the presence of the genus in the hands of the workers while also showing their enterotoxigenic potential. In a study by Rossi Júnior et al. (2001), *A. hydrophila*, *A. caviae* and other atypical strains were isolated from different products and locations in a beef slaughterhouse involving among other types of samples, swabs of the hands of handlers before and during the workday. Also in a slaughterhouse, Martineli et al. (2011) obtained 53.3% of positive samples for the bacteria from the hands of workers.

Regarding the equipment used in the manufacture of different cheeses, only one sample from the artisanal cheese production was positive. The species *A. sobria* and *A. caviae* were isolated with an average population of  $1.0 \times 10^1$  UFC/cm<sup>2</sup>. It is evident, therefore, the importance of the surfaces as contamination sources of the final product and the need to clean and sanitize them properly. It is also noteworthy the possibility of *Aeromonas* to form biofilms on water pipes in the farms, on the milking equipment, industrial utensils or pipe surfaces that become sources of contamination of milk and its derivatives (BOARDI et al. 2009).

*Aeromonas* spp. was not isolated in the production environment of the Colonial cheese; however, the industrial environment was positive for the agent. *A. caviae* was isolated in 20.0% of samples from the environment (population of  $2.1 \times 10^1$  UFC/plate). Studies by Rossi Júnior et al. (2006) and Martineli et al. (2010) also showed the importance of the environment as a possible source of contamination in the industry (slaughterhouse).

The milk used as raw material for the manufacturing of Colonial cheese was not pasteurized; it was heated at average temperature of 34.6°C for 15 minutes for the addition of coagulation enzyme. Under these conditions, *A. hydrophila*, *A. caviae* and *A. veronii* were isolated from both heated and coagulated milk. According to Janda & Abbott (2010), these three species are responsible for the vast majority ( $\geq 85\%$ ) of human infections attributed to the agent.

In contrast, pasteurized raw material was used in the industrial production of Frescal Minas cheese. The genus is sensitive to thermal processing; therefore, the fact that one of the 25 samples of pasteurized milk was positive to the bacteria is believed to be related with recontamination. This hypothesis was also raised by Kirov et al. (1993), who found that 60.0% of raw

milk samples were positive for *Aeromonas* spp. and only 4.0% of pasteurized milk.

Also in the industry, 100.0% of the samples of coagulated milk, molded curd and cheese ready for consumption were negative for *Aeromonas* spp. In the Colonial cheese ready for consumption the agent also was not isolated. The absence of the bacteria in the final stages of the industrial production and the finished product can be justified by the pasteurization process and the low level of contamination observed in the other possible sources of the micro-organism, such as the environment and the hands of the workers. For the Colonial cheese, even though the agent had been isolated from all other production stages, the maintenance of the cheese at room temperature until commercialization may have favored the multiplication of different kinds of microorganisms and, if so, competition may have hindered the isolation of *Aeromonas* in the cultures. The results can also be associated with the stability and viability of the bacteria in food, which is favored by neutral pH, moisture and optimum temperature of 28°C, despite having a wide tolerance range (ABBOTT et al., 2003; DASKALOV 2006). It is possible then that the unfavorable pH of cheese ready for consumption, contributed to the non-isolation of the agent.

The percentage of samples positive for *Aeromonas* spp was significantly different ( $p < 0.01$ ) between the manufacturing establishments. The industrial production, subjected to permanent sanitary-hygienic control, had four sources of contamination and spreading of the agent out of the ten steps studied of the Frescal Minas cheese flowchart production. In comparison, during the artisanal manufacturing of the Colonial cheese, made by hand, the agent was isolated in seven out of the nine points analyzed. It should be emphasized the increased risk that informal food production poses for the consumer population.

## CONCLUSION

These results clearly show that during the production steps of Colonial and Minas Frescal cheeses, there are different sources of infection and dissemination of *Aeromonas* spp. indicating the risk to public health, even though the agent was not present in the final product. In the industrial production, raw and pasteurized milk, workers and the environment acted as sources of the micro-organism. The supply water, raw and heated milk, the hands of workers, utensils and coagulated milk presented the higher risks as sources of contamination and spreading of the bacteria during Colonial cheese production. Noteworthy are the high counts obtained for the raw material used for producing different types of cheese.

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