

Research Paper

## Microbiological aspects of the biofilm on wooden utensils used to make a Brazilian artisanal cheese

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### Abstract

The artisanal Minas cheese is produced from raw cow's milk and wooden utensils were employed in its manufacture, which were replaced by other materials at the request of local laws. This substitution caused changes in the traditional characteristics of cheese. Due to the absence of scientific studies indicating the microbial composition of biofilms formed on wooden forms, tables and shelves used in these cheese production, the present work evaluated the counts of *Staphylococcus aureus*, *Escherichia coli*, coliforms at 32 °C, yeasts, presumptive mesophilic *Lactobacillus* spp. and *Lactococcus* spp. in these biofilms, milk, whey endogenous culture and ripened cheese in two traditional regions: Serro and Serra da Canastra. Also, we checked for the presence of *Salmonella* sp. and *Listeria monocytogenes* in the ripened cheeses. The ultra structure of the biofilms was also assessed. Counts above legislation (> 2 log cfu/mL) for the pathogens evaluated were found in milk samples from both regions. Only one shelf and one form from Serro were above limits proposed (5 cfu/cm<sup>2</sup> for *S. aureus* and *E. coli* and 25 cfu/cm<sup>2</sup> for coliforms) in this study for contaminants evaluated. In Canastra, few utensils presented safe counting of pathogens. There was no *Salmonella* sp. and *Listeria monocytogenes* in the cheeses after ripening. Thus, the quality of the cheese is related to improving the microbiological quality of milk, implementation and maintenance of good manufacturing practices, correct cleaning of wooden utensils, and not its replacement.

**Key words:** biofilm, artisanal minas cheese, lactic acid bacteria, Serro, Serra da Canastra.

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### Introduction

Wood has been historically used in manufacturing utensils for making food such as vinegar (Solieri and Giudici, 2008), cider (Del Campo *et al.*, 2003) and cheese, especially those with Protected Designation of Origin (PDO) status commercialized in European countries (Casalta *et al.*, 2009; Mariani *et al.*, 2007; Lortal *et al.*, 2009).

In the manufacturing process, artisanal Minas cheese a traditional Brazilian cheese, made from raw cow's milk added rennet and whey endogenous culture (EC) comes into contact with wooden forms and tables during shaping, manual pressing and standing on wooden shelves for ripening (EMATER, 2004). The accumulation of organic matter

in these utensils, among which proteins, carbohydrates and other compounds present in the milk, contributes to biofilm formation (Capdeville and Nguyen, 1990; Trulear and Characklis, 1982). The biofilm on utensils in the environment of cheese dairies is commonly formed by microbial components from the milk microbiota, such as yeasts and lactic acid bacteria (LAB), and also by microorganisms in the air and on the hands of cheese makers, such as filamentous fungi and pathogens. This biofilm can detach from the surface of wooden utensils becoming part of the EC microbiota ("pingo" that means drop) that will be consequently present in the cheese contributing to its ripening (Lortal *et al.*, 2009). The pingo is collected daily after cheese production and remains refrigerated until the next batch. Laws of the State of Minas Gerais - the Brazilian state that produces

this cheese - established that the wooden forms and tables were replaced by other materials (Minas Gerais, 2002a) and directed cheese makers to the standards of good manufacturing practices (GMP). Wooden forms were replaced by polypropylene or high density polyethylene forms, while wooden tables were substituted for slate or stainless steel. The replacement of wooden utensils by other materials changed the characteristics of the cheese. According to cheese makers, these new materials do not contribute to the syneresis of cheese, affecting the traditional flavor and texture. Furthermore, European studies indicate that the biofilm formed on wooden utensils have a role in the characteristics of ripened cheese that have PDO, such as Raguano (Licitra *et al.*, 2007; Lortal *et al.*, 2009) and Reblonch de Savoie (Mariani *et al.*, 2007).

The ways of manufacturing artisanal Minas cheese in two traditional producing regions (Serro and Serra da Canastra) are similar for milking and adding EC and rennet to milk. During shaping, the cheese makers in Serro press the curd inside the forms manually. On the other hand, in Canastra, the curd is placed inside tissues that aid in pressing. For this reason, the cheese produced in Canastra initially have lower moisture content than that from Serro, thus Canastra cheese needs more time to ripening, as verified by Dores (2007). In the traditional method, after shaping, artisanal Minas cheese is placed on wooden shelves for ripening (Minas Gerais, 2002b). During this process, different physical and chemical changes such as reduction of pH (Beuchat and Golden, 1989), reduced redox potential (Crow *et al.*, 1995) and decrease in water activity (Brown, 1976) restrict pathogen growth and allow the development of LAB that are better adapted to these conditions. At different moments, LAB and yeasts originating from the EC and/or biofilm from wooden utensils produce enzymes, organic acids and other antimicrobial compounds such as peptides and bacteriocins that aid in product safety (González *et al.*, 2007). In addition, these modifications are essential for defining the aroma, flavor and texture of the cheese.

Since wooden tables and forms have been removed from the production of artisanal Minas cheese, which has subsequently altered its characteristics, and given the inexistence of scientific studies indicating the composition of microbial biofilms formed on these utensils, the objectives of this study were to get insights and present an overview of the microbial situation of wooden utensils by pathogen enumerations, and also enumeration of microorganisms involved on ripening of the artisanal Minas cheese like yeasts and the two principal genera of LAB found in these cheese mesophilic *Lactobacillus* spp. and *Lactococcus* spp. It were also evaluated the counts of these microorganisms on milk, EC and ripened cheese, as well as checking the ultra structure of the utensils biofilm.

## Materials and Methods

### Milk, endogenous culture, cheese and biofilm sampling

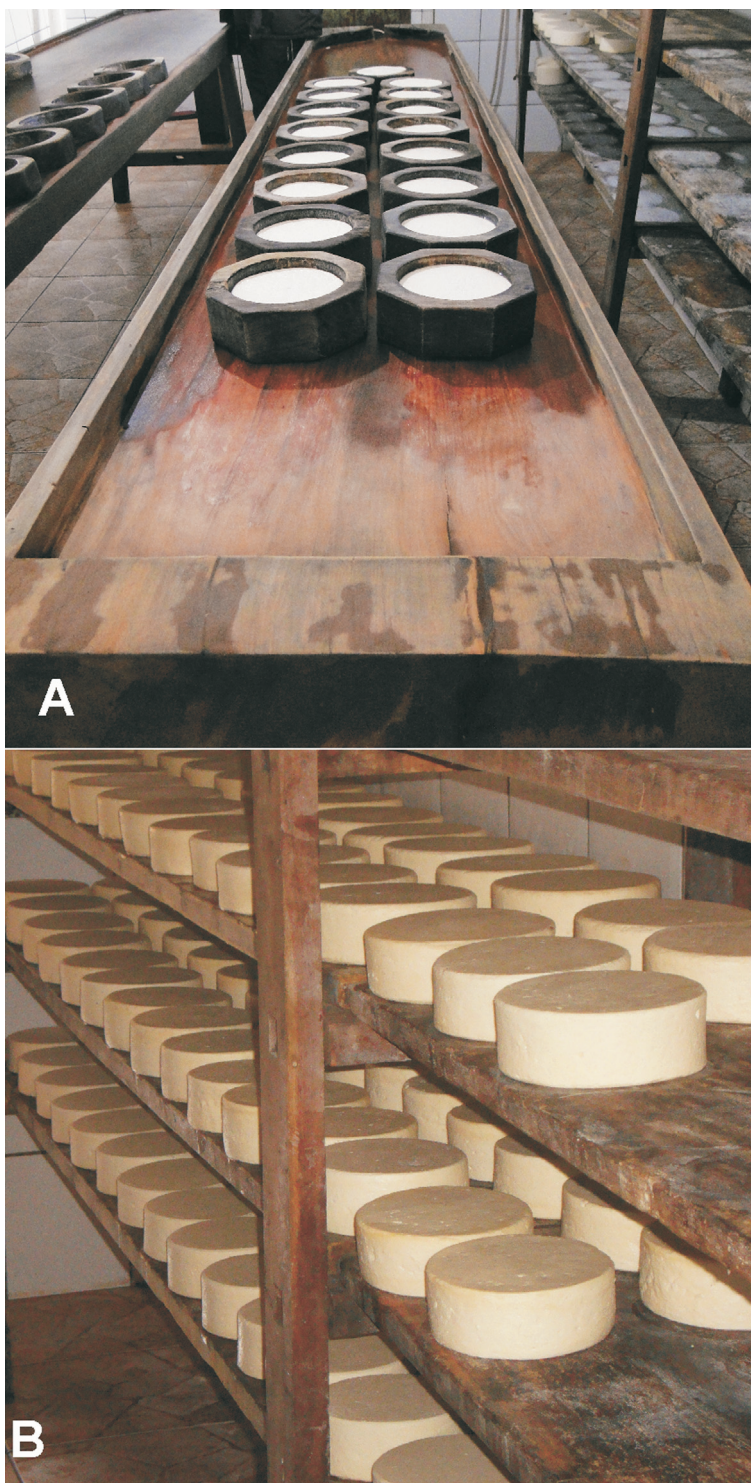
The number of utensils sampled represents the remaining wooden utensils still in use for artisanal Minas cheese making while the other producers have already replaced their utensils. These utensils are used and washed daily with water and have been used at least for five years.

Samples to evaluate biofilm on wooden utensils were obtained by the swab method (APHA, 2001), delimiting the sampling area with a sterile template of 10 cm x 10 cm dimensions to collect on the shelves and tables and 2 cm x 2 cm on the forms. Samples were collected from the surfaces of three tables, forms and shelves in Serro region, and three tables and shelves in Serra da Canastra region (Figures 1A and B). In the Serra da Canastra are no longer used wooden forms. The same microbial groups accessed in the biofilms were evaluated in three samples of milk before addition of EC, three samples of EC and ripened cheese in each region. The samples of milk and EC collected were the same used in the manufacture of cheeses evaluated. For each table it was sampled three areas of 100 cm<sup>2</sup> and plated separately to evaluate the biofilm, a sample being taken from the central portion and two others from their extremities. The average of the counts from three points was obtained. A sample of 100 cm<sup>2</sup> was taken from each shelf. A swab was used to collect four samples of 4 cm<sup>2</sup> from each form. After sampling, each swab was placed in a flask containing 10 mL of 0.12% phosphate buffer solution, sterilized by autoclaving (121 °C for 15 min), labeled and then stored on ice until the time of microbiological evaluations. Aliquots of 1 mL of each biofilm sample was diluted in 9 mL of 0.12% phosphate buffer, followed by serial decimal dilutions before plating in depth in the media described below.

### Microbiological analysis

One-mL aliquots of milk and EC were diluted in 9 mL of 0.12% phosphate buffer solution, followed by serial decimal dilutions before plating. Portions of 25 g were taken from each ripened cheese for homogenization in 225 mL of 0.12% phosphate buffer solution using Stomacher 400 Bagmiser® (Model VW, France) for 2 min at low speed. Serial decimal dilutions were made in samples previously homogenized before plating in depth.

The microorganisms quantified were: total aerobic mesophiles, yeasts, presumptive mesophilic *Lactobacillus* spp. and *Lactococcus* spp., *Staphylococcus aureus*, *Escherichia coli* and coliforms at 32 °C (designated here simply as coliforms). Aerobic mesophiles were grown on plate count agar (Difco, Lawrence, Kansas, USA) incubated at 30 °C for 48 h. Yeasts were counted after aerobic incubation at 28 °C for 5 days on potato dextrose agar (Difco, Lawrence, Kansas, USA). Mesophilic *Lactobacillus* spp.



**Figure 1** - Wooden table and forms from Serro (A), wooden shelves from Serro (B).

were grown anaerobically in MRS agar (Difco, Lawrence, Kansas, USA; De Man *et al.*, 1960) and *Lactococcus* spp. aerobically in M17 agar (Difco, Lawrence, Kansas, USA; Terzaghi and Sandine, 1975) both incubated at 30 °C for 48 h. *S. aureus*, *E. coli* and coliforms were evaluated in specific Petrifilm® plates (3M, St. Paul, Minnesota, USA; 3M,

2013) and incubated following the manufacturer's protocol. The microbiological evaluation was done in cheeses after ripening for 17 days for Serro region (Martins, 2006) and 22 days for Canastra region (Dores, 2007). All plating was performed in duplicate. To check for the presence of *Salmonella* sp. and *Listeria monocytogenes* in

cheeses ripened for the time periods mentioned above, we used Reveal<sup>®</sup> kits (Neogen, Leshar Place Lansing, Miami, USA), following the manufacturer's protocol, which involves a pre-enrichment step before testing. The microbial counts were evaluated by the method of descriptive statistics.

#### pH and moisture measurement

The pH of EC and of ripened cheeses was measured using specific pH meters. The moisture content of the ripened cheeses was determined according to International Dairy Federation - IDF (IDF, 1982).

#### Scanning electron microscopy

Fragments of wooden utensils (10 x 3 x 1 mm approximately) were obtained for scanning electron microscopy (SEM). Samples from all utensils were fixed in a solution 1:1 (v/v) 4% glutaraldehyde and 4% paraformaldehyde (Karnovsky, 1985) for 12 h. The samples were then rinsed with sodium cacodylate buffer for 12 h and dehydrated in serial ethanol baths (30, 50, 70, 80, 95, 100, 100 and 100%) for 10 min at room temperature. After dehydration, the samples were dried in a critical point dryer with CO<sub>2</sub> (Bal-Tec, Model CPD030, England) and coated with gold for 2 min in a cathode vacuum evaporator (Balzers, Model FDU010, Germany). Observations of samples were made using a LEO1430 scanning electron microscope (Model VP, England) at an accelerating voltage of 20 kV.

## Results

### Count groups related to the microbiota of artisanal Minas cheese

The mean counts of total aerobic mesophiles, yeasts, mesophilic *Lactobacillus* spp. and *Lactococcus* spp. in samples from Serro and Canastra regions are shown in Table 1. The aerobic mesophilic counts in the milk samples of Serro and two from Canastra were above the current legislation (> 5 log cfu/mL; Minas Gerais, 2002b). In the region of Serro, the values obtained for aerobic mesophiles on wooden utensils ranged from 0.96 log cfu/cm<sup>2</sup> in the form and 6.24 log cfu/cm<sup>2</sup> in the shelf. In Canastra, the lowest and highest values of aerobic mesophiles observed on wooden surfaces were 3.5 and 6.24 log cfu/cm<sup>2</sup> in tables; 4.2 and 4.87 log cfu/cm<sup>2</sup> in shelves. The variation in yeast count observed in Canastra was 2.46 log cfu/mL in milk and 5.93 log cfu/g on cheese. In Serro, the highest yeast count was observed on cheese (5.05 log cfu/g) and the lowest on wooden form biofilm (1 cfu/cm<sup>2</sup>).

### Enumeration of pathogens in artisanal Minas cheese and wooden utensils

The mean counts of pathogens in samples from Serro and Serra da Canastra are shown in Table 1. The counts of *S. aureus* in all samples of ripened cheese from Canastra

and two from Serro were below the maximum legal limit ( $\leq 2$  log cfu/g; Minas Gerais, 2002b). However, counts of this pathogen in two milk samples from each region were above the current standard. The ripened cheese counts from Serro were also safe for *E. coli* and coliforms ( $\leq 2$  and  $\leq 2.7$  log cfu/g, respectively). In Canastra, only one cheese had safe counts of these contaminants. Only one milk sample from Serro showed high *E. coli* counts (> 3.5 log cfu/mL), whereas milk samples from Canastra exhibited safe levels of this pathogen ( $\leq 2$  log cfu/mL). The current legislation does not define the limit of milk coliforms and therefore, we established the value of 500 cfu/mL (2.7 log cfu/mL), representing half of what is proposed for coliforms in cheese. Thus, one milk sample from Canastra and two from Serro were contaminated by coliforms above 3.6 log cfu/mL. As there is no legislation establishing limits of microbial indicators of safety for the EC used in the manufacture of artisanal Minas cheese, the same standards for milk were adopted, considering low contamination values  $\leq 2$  log cfu/mL for *S. aureus* and *E. coli* and  $\leq 2.7$  log cfu/mL for coliforms. This way, only one EC sample from Canastra did not have safe levels. In Serro one EC sample exhibited 2.7 log cfu/mL for *S. aureus*; two were higher than 2.6 log cfu/mL for *E. coli*, and one higher than 2.8 log cfu/mL for coliforms. For wooden surfaces, we proposed the limit for *S. aureus* and *E. coli* as 20 times lower (5 cfu/cm<sup>2</sup> or 0.7 log cfu/cm<sup>2</sup>) than that established by law for artisanal Minas cheese. In addition, the proposed value corresponds to that 10 times lower than the one the World Health Organization recommends for aerobic mesophiles on surfaces (Silva Jr., 2001). To be less related to pathogens we suggested the limit for coliforms on the wooden surfaces five times the limit proposed for *E. coli* (25 cfu/cm<sup>2</sup> or 1.4 log cfu/cm<sup>2</sup>). The wooden forms from Serro had low counts of *E. coli* and coliforms; only one showed 1.14 log cfu/cm<sup>2</sup> for *S. aureus*. All tables from Serro had low counts for contaminants evaluated. In Canastra, a table had counts lower than that proposed for *S. aureus* and *E. coli*, while two of them had low counts of coliforms. Only one shelf from Serro had above-limit counts for coliforms while the other ones had no coliforms. In Canastra, two shelves were contaminated with *S. aureus* and one with *E. coli*. There was no *Salmonella* sp. or *Listeria monocytogenes* in samples of ripened cheese from both regions.

### pH and moisture of the cheese

The pH ranged from 4.58 to 4.77 and 4.57 to 4.88 for Serro and Canastra ripened cheeses, respectively. The pH of EC samples ranged from 4.41 to 4.74 in Serro and 5.53 to 5.67 in Canastra. The moisture content of the cheese samples from both regions after the ripening period of 17 and 22 days for Serro and Canastra, respectively, were below the 45.9% specified by legislation (Minas Gerais, 2008), averaging 37.3% in Canastra region and 37.4% in Serro region.

**Table 1** - Log<sub>10</sub> counts (mean ± SD) of microorganisms involved in the safety and ripening of artisanal Minas cheese made in Serro and Serra da Canastra regions.

| Microorganisms               | Regions     | cfu/mL      |             | cfu/cm <sup>2</sup> |             |             | cfu/g       |             |             |
|------------------------------|-------------|-------------|-------------|---------------------|-------------|-------------|-------------|-------------|-------------|
|                              |             | Milk        | EC          | Form                | Table       | Shelf       | Cheese      |             |             |
| Aerobic mesophiles           | Serro       | 6.64 ± 0.44 | 7.48 ± 0.82 | 2.52 ± 1.44         | 4.29 ± 1.31 | 4.69 ± 1.38 | 8.00 ± 0.51 |             |             |
|                              | Canastra    | 6.23 ± 1.27 | 5.81 ± 0.50 |                     |             |             | 4.58 ± 1.46 | 4.56 ± 0.34 | 8.18 ± 0.53 |
|                              | Legislation | ≤ 5.00      |             |                     |             |             |             |             |             |
| Yeasts                       | Serro       | 3.13 ± 1.18 | 4.48 ± 0.32 | 1.03 ± 1.39         | 2.98 ± 0.23 | 3.21 ± 0.39 | 5.00 ± 0.03 |             |             |
|                              | Canastra    | 3.93 ± 1.54 | 3.85 ± 0.48 |                     |             |             | 3.27 ± 0.21 | 3.25 ± 0.65 | 5.21 ± 0.63 |
| <i>Lactobacillus</i> spp.    | Serro       | 4.57 ± 0.46 | 6.92 ± 1.14 | 2.59 ± 1.20         | 4.01 ± 0.52 | 3.99 ± 0.42 | 8.00 ± 0.40 |             |             |
|                              | Canastra    | 5.65 ± 1.33 | 5.42 ± 0.50 |                     |             |             | 4.26 ± 1.01 | 4.35 ± 0.57 | 8.21 ± 0.33 |
| <i>Lactococcus</i> spp.      | Serro       | 6.02 ± 0.44 | 7.50 ± 0.56 | 2.27 ± 1.71         | 4.58 ± 0.69 | 4.68 ± 1.07 | 8.20 ± 0.49 |             |             |
|                              | Canastra    | 6.31 ± 1.38 | 5.86 ± 0.39 |                     |             |             | 4.39 ± 1.14 | 4.53 ± 0.35 | 8.09 ± 0.27 |
| <i>Staphylococcus aureus</i> | Serro       | 2.25 ± 2.00 | 0.90 ± 1.56 | 0.38 ± 0.66         | 0.15 ± 0.15 | 0.16 ± 0.28 | 2.10 ± 1.24 |             |             |
|                              | Canastra    | 2.48 ± 1.68 | 1.59 ± 0.36 |                     |             |             | 0.73 ± 0.68 | 0.85 ± 0.73 | 0.95 ± 0    |
|                              | Legislation | ≤ 2.00      |             |                     |             |             |             |             |             |
| <i>Escherichia coli</i>      | Serro       | 1.70        | 1.58        | 0 ± 0               | 0.07 ± 0.12 | 0.27 ± 0.47 | 1.30 ± 0.33 |             |             |
|                              | Canastra    | 1.13 ± 0.31 | 0.97 ± 0.03 |                     |             |             | 0.74 ± 1.29 | 0.47 ± 0.45 | 3.10 ± 1.02 |
|                              | Legislation | ≤ 2.00      |             |                     |             |             |             |             |             |
| Coliforms                    | Serro       | 3.08 ± 1.53 | 1.83 ± 1.59 | 0 ± 0               | 0.33 ± 0.30 | 0.66 ± 1.14 | 1.50 ± 0.70 |             |             |
|                              | Canastra    | 2.53 ± 0.98 | 1.57 ± 1.03 |                     |             |             | 2.23 ± 1.23 | 1.17 ± 0.12 | 3.71 ± 1.02 |
|                              | Legislation |             |             |                     |             |             |             |             |             |

### Ultra structure of the biofilm from utensils

The electron micrographs showed that surfaces of the utensils were almost completely covered by a diversified ecosystem consisting of cocci, bacilli, yeasts and filamentous fungi (Figure 2). Tables from both regions showed a diversity of microorganisms, although filamentous fungi were observed more frequently on the samples from Serro and rarely on those from Canastra. Cocci were the predominant shape observed in all wooden utensils from both regions. Moreover, bacilli were seen more frequently on utensils from Serro region than those from Canastra. It is suggested the presence of exopolysaccharides where the cocci are inserted (Figure 2D).

### Discussion

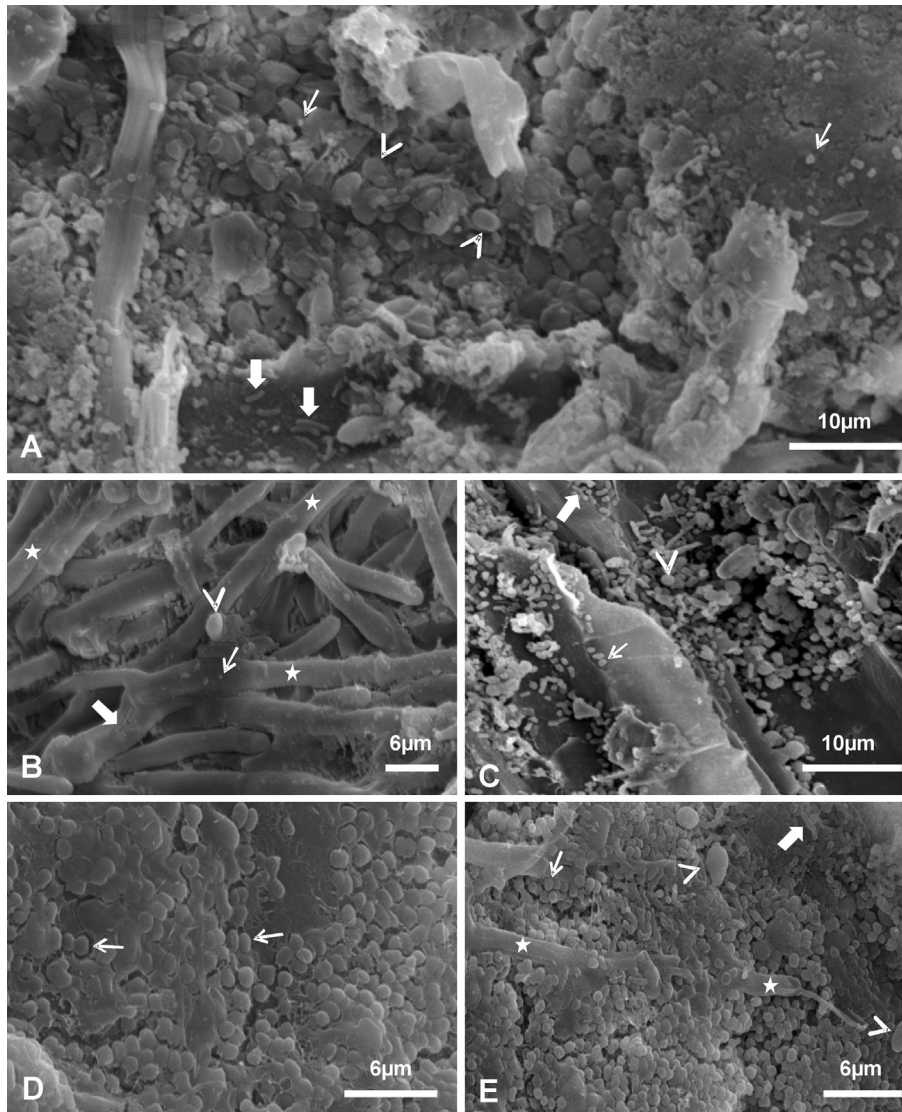
This study describes for the first time the microorganisms in biofilms of wooden utensils involved in the safety of artisanal Minas cheese as well as those responsible for its ripening. And yet, this work assessed for the first time the ultra structure of these biofilms.

The high count of aerobic mesophiles in milk and EC samples may be related to the corresponding numbers of mesophilic lactobacilli and lactococci in these raw materials. The counting of aerobic mesophiles on the surfaces of tables and shelves from the two regions confirms the existence of a biofilm with diverse microbiota, as observed by

Lortal *et al.* (2009) in biofilm formed on wooden vats used in the manufacturing of Ragusano cheese.

Yeasts present in biofilms can act in two ways in artisanal cheese. Firstly, they may cause spoilage of the product, resulting in an undesirable flavor, discoloration, gas production and changes in texture (Fleet, 1990). However, they may exert beneficial effects through proteolytic and lipolytic activities, leading to the formation of flavor during ripening (De Freitas *et al.*, 2009; Fadda *et al.*, 2004). Borelli *et al.* (2006) obtained yeast counts above 7 log cfu/g in cheese from Canastra after five days of ripening and among the several species present, the main were *Candida catenulata*, *Debaryomyces hansenii*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*. This last one is able to assimilate and ferment lactose (Fleet, 1990) and thus, should be involved synergistically with LAB during the initial acidification of the cheese. This counting can be related to the insufficient time of ripening for the cheese from Canastra, which at 22 days, comes close to those obtained in our study (5.2 log cfu/g).

Knowing that the pH influences the adherence capacity of a microorganism (Mafu *et al.*, 2011) and low pH reduces the rate of adherence of *S. aureus* (Zmantar *et al.*, 2010) and *E. coli* (Mafu *et al.*, 2011), the low counts of all contaminants in all tables from Serro, *S. aureus* in one table and *E. coli* in two tables from Canastra can be attributed to the acidic condition of the surfaces, whose pH must be near



**Figure 2** - Scanning electron microscopy of biofilms on wooden utensils from Serro: table (A), form (B) and shelf (C); Canastra: table (D) and shelf (E). Cocci (thin arrows), bacilli (large arrows), yeast (arrowheads) and filamentous fungi (stars).

the average pH of the EC from Serro (4.54) and Canastra (5.58). In addition, factors such as the production of exopolysaccharides (Kim *et al.*, 2009) and bacteriocins by LAB (González *et al.*, 2007), competition for nutrients and adhesion sites should be considered. According to Carmo *et al.* (2002), food poisoning caused by *S. aureus* enterotoxins occur when the counts of this microorganism exceeds 6 log cfu/g in food. Therefore, it is essential to control this pathogen numbers in utensils and especially raw materials. The counts observed in the milk samples indicate unsatisfactory GMP. Thus, the microbiological quality of milk should receive special attention, since this is the main source of microorganisms that will form biofilms on the utensils and direct their safe use.

Although ripening is known to reduce pathogens, it appears that the processing starting with a microbiological quality raw material favors ripening and keeps pathogen

counts below legally safe levels. When the counts of *E. coli* in milk were compared to those in ripened cheese, it was found that even when milk samples from Canastra had low counts of *E. coli*, two cheeses from this region presented counts ( $> 3.3$  log cfu/g) above the safety standards for this pathogen. This indicates that milk contributes to the contamination existing on wooden utensils, and consequently the cheese contamination. High counting of *S. aureus* and coliforms found in two of the Canastra milk samples should have a direct influence on the elevated counting obtained on the utensils. However, *S. aureus* were at low levels ( $< 1$  log cfu/g) in cheeses from Canastra, while *E. coli* and coliforms count remained high, suggesting inappropriate cleaning of the shelves. Furthermore, tables from Canastra contained slots across the surface, making it difficult to clean and enabling organic matter accumulation and microbial growth. The cheese makers from Canastra region

should give a good surface finish of the tables, so that they become as smooth as possible, facilitating cleanup. Therefore, producers in both regions must apply correctly the GMP, avoiding contamination of the milk.

The high counts of mesophilic *Lactobacillus* spp. and *Lactococcus* spp. in biofilms on tables and shelves from both regions are desirable, since these microorganisms can be released from the biofilm and join those existing in milk (Lortal *et al.*, 2009) and the EC that will be used for the next batch. LAB are responsible for producing the ripened cheese flavor (Awad *et al.*, 2007; Randazzo *et al.*, 2010) by the acidification and production of antimicrobial compounds (Kim *et al.*, 2009; Settanni *et al.*, 2011), which inhibit the growth of pathogens and control the multiplication of spoilage and contaminating microorganisms.

The variability of shapes observed by SEM may be related to the various methods of cleaning used by cheese makers in each region. Although this work was not done to quantify filamentous fungi in biofilms, they were nonetheless observed in the micrographs of some tables and shelves from the two regions and forms from Serro (Figure 2B), showing the diversity of microorganisms that may constitute these biofilms. The lower microbial counts obtained in a few utensils alone do not indicate the formation of a biofilm. However, micrographs showed the presence of such a structure with microorganisms adhered to the surfaces.

To sum up, the results of this study suggest that wooden utensils could continue to be used, as long as the cheese makers do their cleaning properly, especially those of the Serra da Canastra, due to surface irregularities. It is important to emphasize here that milk should be at lower degree of contamination, which would favor the ripening of cheese, guaranteeing the consumer a safe product and maintaining its traditional sensory characteristics. More studies should be done to verify the complete microbiota existing in these biofilms, and thus understand the role of each microorganism in the process of ripening and safety of artisanal Minas cheese.

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