

COAGULASE POSITIVE STAPHYLOCOCCI ENUMERATION AND ENTEROTOXINS DETECTION IN MILK AND DAIRY PRODUCTS FROM CENTRAL ITALY

L. IANNETTI¹, P. VISCIANO², C. MARFOGLIA¹, G. IANNITTO¹, G. PARISCIANI¹,
M. SERICOLA¹, D. PETRONE¹, M.S. MANGIERI², F. POMILIO¹ and M. SCHIRONE^{*2}

¹Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Via Campo Boario,
64100 Teramo, Italy

²Faculty of Bioscience and Technology for Food, Agriculture and Environment,
University of Teramo, Via R. Balzarini 1, 64100 Teramo, Italy

^{*}Corresponding author: Tel.: +390861266911

E-mail address: mschirone@unite.it

ABSTRACT

This study aims at enumerating coagulase positive staphylococci (CPS) in 404 samples of milk and dairy products collected in own-checks or during the official controls from different dairy industries located in Central Italy. These microorganisms were enumerated using ISO 6888-2:1999/Amd. 1:2003 and only when they exceeded 10^5 CFU/g, the presence of any of the seven more common staphylococcal enterotoxins (SEA, SEB, SEC₁, SEC₂, SEC₃, SED and SEE) was also investigated. Own-checks samples resulted always below the detection limit, whereas among those collected by the competent authorities in the framework of official controls, *provola* (100%) and *mozzarella* (22.9%) samples were positive to CPS, with mean values of 1.8×10^5 and 2.8×10^5 CFU/g respectively. Such values exceeded the maximum limits set by Commission Regulation (EC) No. 2073/2005, resulting in a request of hygiene improvements in the first case; in the second case, the presence of staphylococcal enterotoxins in 8 (2.7%) *mozzarella* samples out of 298 investigated cheese products was also observed, resulting in their withdrawn from the market. Therefore, this study aims at highlighting that monitoring of CPS incidence in dairy products and subsequent testing of cheeses for enterotoxins when appropriate represent an important tool for public health in order to avoid the occurrence of foodborne outbreaks.

Keywords: Coagulase positive staphylococci, milk, dairy products, process-hygiene criteria, staphylococcal enterotoxins

1. INTRODUCTION

Italian cheese production boasts a great variety of dairy products ranging from fresh to ripened cheeses such as *mozzarella*, *pecorino* and *Parmigiano Reggiano*. Their quality is closely associated with parameters such as the territory of production, pedoclimatic characteristics, and anthropic components (SCHIRONE *et al.*, 2012). Abruzzo and Molise regions located in Central Italy have no cheeses with protected designation of origin (PDO) or protected geographical indication (PGI), except for *caciocavallo silano* and *mozzarella di bufala campana* for Molise region, as reported in the publicly-accessible updated register of PDO and PGI of Regulation (EU) No. 1151/2012 (EU, 2012). In contrast, according to the Italian Decree No. 350 dated September 8, 1999 (MIPAAF Decree, 1999), 14 and 12 traditional cheeses are reported as traditional food products made in Abruzzo and Molise regions respectively. As far as such traditional cheeses are concerned, raw milk microbiota rather than microorganisms deriving from the dairy environment or equipment can affect the safety of the end product (SÁNCHEZ-GAMBOA *et al.*, 2018; SCHIRONE *et al.*, 2018).

Staphylococci are widespread in nature, being found on the skin and mucous membranes of animals and humans, but also in the environment (RUIZ *et al.*, 2016). Coagulase positive staphylococci (CPS) are the most common causative agents of subclinical mastitis in dairy cows (RAJIC-SAVIC *et al.*, 2015). Several studies (JORGENSEN *et al.*, 2005; NEDER *et al.*, 2011) reported that some strains isolated from bulk milk can produce enterotoxins and, as such, they are considered biological hazards for the dairy industry. Among them, *Staphylococcus aureus* is one of the most important disease-causing agents of foodborne intoxication in humans caused by the consumption of dairy products but, since it is also found in 30-80% of human population, the personnel working in a food industry can contaminate food with unhygienic food handling and manufacturing practices (SOLTAN DALLAL *et al.*, 2016).

According to Commission Regulation (EC) No. 2073/2005 (EC, 2005) and further amendments, CPS should be investigated in different categories of cheese, both ripened and unripened, made from raw or pasteurized milk, or after a stronger heat treatment, for process control purpose. The process-hygiene criteria established by the above quoted Regulation differ on the basis of cheese categories and can be applied at the end of the manufacturing process or at the time when the number of staphylococci is expected to be the highest. The current legislation on microbiological criteria requires that only when such values exceed 10^5 CFU/g the cheese batch must be analyzed for staphylococcal enterotoxins, even if some authors reported that levels of *S. aureus* ranging from not detectable to 1.5×10^{10} CFU/g, with a median value of 3×10^5 CFU/g, could cause foodborne outbreaks (WALLIN-CARLQUIST *et al.*, 2010). Their formation is influenced by some parameters, such as temperature, pH, water activity, redox potential and bacteria antagonism (SCHELIN *et al.*, 2011). The most common detected staphylococcal enterotoxins named as classical (SEA, SEB, SEC, SED and SEE) can cause several symptoms at rapid onset (2-8 h), including hypersalivation, nausea, vomiting, abdominal cramping and diarrhoea, after the ingestion of contaminated food (MARTINS *et al.*, 2014; MEHLI *et al.*, 2017). Staphylococcal food poisoning usually resolves within 24-48 h with no specific treatment; but it can be more severe or even fatal when occurring in infants, elderly or immune-compromised people (ROLA *et al.*, 2016).

The aim of the present investigation was the enumeration of CPS in milk and dairy products produced in dairy industries located in Abruzzo and Molise regions, Central Italy, in order to check the process-hygiene criteria established by Regulation (EC) No. 2073/2005 and to detect staphylococcal enterotoxins only in not compliant samples.

2. MATERIAL AND METHODS

A total of 404 samples of milk and dairy products were obtained from different dairy industries located in Abruzzo and Molise regions (Central Italy) in the framework of own-checks (n=118) and official controls (n=286).

The milk samples (n=91) were differentiated into raw milk (n=14), bulk milk (n=28), whole milk (n=21), low-fat milk (n=19) and skimmed pasteurized milk (n=9). Among the dairy products, butter (n=9) and cream (n=6) were sampled. All the other remaining dairy products belonged to different categories of cheese (n=298) and could be grouped according to the process-hygiene criteria of Commission Regulation (EC) No. 2073/2005 – namely raw milk cheese (n=18), ripened cheese from pasteurized milk (n=36) and unripened cheese from pasteurized milk (n=244). Among the latter, only *caciotta* samples were produced from raw cow' or sheep's milk; while most of cheese samples were represented by unripened cheeses, made from pasteurized cow' or water buffaloes' milk, usually "pasta filata" fresh cheeses (i.e. *burrata*, *mozzarella*, *provola*, *scamorza* and *fiordilatte*), semi-soft cheeses and ricotta. Ripened cheeses were also from pasteurized cow' or sheep's milk and included semi-hard cheeses and grated cheese.

All samples were analyzed at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise – that is, the laboratory in charge for official controls in those regions, which also tested samples taken in the framework of own-checks from some local dairy industries. Samples collected over a 18-month period, including the whole year 2017 and the first 6 months of the year 2018, were involved in the present study. They were investigated for CPS enumeration by the analytical reference method ISO 6888-2:1999/Amd. 1:2003. Such a method is based on the use of Rabbit Plasma Fibrinogen Agar that allows the easy differentiation of coagulase positive and negative colonies with no need of confirmatory steps (VIÇOSA *et al.*, 2009). Briefly speaking, duplicate poured plates of the quoted medium were prepared and inoculated using decimal dilutions of the test sample (or initial suspension, if solid) and then incubated at 37°C for 18-24 h or more, if needed. The calculation of CPS per g of a sample was made counting typical colonies on petri dishes.

According to prescriptions from Commission Regulation (EC) No. 2073/2005, samples with CPS counts exceeding 10⁵ CFU/g were also analyzed for the detection of staphylococcal enterotoxins using the VIDAS® Staph enterotoxin II (SET2) kit (Biomerieux, France) according to the FDA Bacteriological Analytical Manual (BAM), 8th Edition, chapter 13A (revision March 2011). Such a method allows the detection of any of the seven more common staphylococcal enterotoxins (SEA, SEB, SEC₁, SEC₂, SEC₃, SED, SEE). In a few words, toxins were extracted from the test samples (25 g or mL) adding extraction buffer, blending and centrifuging according to the manufacturer's instructions. The supernatant was pumped through moistened absorbent cotton placed in a syringe, using the plunger; then 500 µL of the filtrate was placed in the sample well of the VIDAS SET2 reagent strip and the assay was finally performed with a MINI VIDAS® instrument (Biomerieux, France).

3. RESULTS AND DISCUSSION

Most dairy products, including semi-hard and grated cheese, resulted below the detection limit of CPS (<1 CFU/mL or <10 CFU/g). Table 1 shows the results reported in the different cheese types, in relation to the presence of unsatisfactory results of CPS enumeration according to the Regulation (EC) No. 2073/2005.

Table 1. Number of cheese samples (n) and unsatisfactory CPS enumeration for each cheese type according to the category of the Regulation (EC) No. 2073/2005. Limits are expressed in parentheses; the lowest is allowed only for 2 samples out of 5, the highest is never allowed.

Samples	Raw milk cheeses (10^4 - 10^5 CFU/g)		Ripened cheeses* (10^2 - 10^3 CFU/g)		Unripened cheeses* (10 - 10^2 CFU/g)	
	n	Unsatisfactory	n	Unsatisfactory	n	Unsatisfactory
<i>Caciotta</i>	18	0**				
<i>Ricotta</i>					10	0
Soft cheese					10	0
Semi-soft cheese					5	0
Semi-hard cheese			27	0		
Grated cheese			8	0		
<i>Burrata</i>					8	0
<i>Mozzarella</i>					35	8
<i>Provola</i>					5	5
<i>Scamorza</i>					73	0
Other "Pasta filata" cheeses					99	0

*made from milk or whey that has undergone pasteurization or a stronger heat treatment;

**CPS detected below regulatory limits

Coagulase positive staphylococci were only found in samples from official controls, while own-checks were always below the detection limit.

The results of CPS in milk samples were below the detection limit (<1 CFU/mL) except for 5 samples of raw milk collected in the framework of official controls from a small dairy industry in Molise region, that showed values ranging from 2.9×10^3 to 3.5×10^3 CFU/mL with a mean value of 3.5 Log CFU/mL (Fig. 1). The microbial contamination of raw milk may originate from animals, due to endogenous or udder infection, or from faeces and skin, and/or from the environment during milking or storage (VERRAES *et al.*, 2014).

Only some categories of unripened cheese resulted positive to CPS – namely *caciotta*, *provola* and *mozzarella* samples. In particular, the values of CPS ranged from 4.8×10^3 to 5.4×10^3 CFU/g in 5 (33.3%) *caciotta* samples with a mean value of 3.7 Log CFU/g. However, they did not exceed the limits (10 - 10^5 CFU/g) of process-hygiene criteria established by Commission Regulation (EC) No. 2073/2005 for cheeses made from raw milk. All (100%) *provola* samples resulted positive to CPS, ranging from 1.2×10^3 to 2.2×10^3 CFU/g with a mean value of 2.3 Log CFU/g (Fig. 1). These values exceeded the limits (10 - 10^5 CFU/g) for unripened cheeses made from pasteurized milk and therefore process hygiene improvements were required to the involved dairy industry. In addition, 8 (22.9%) samples of *mozzarella* out of 35 exceeded the regulatory limits up to a maximum value of 3.5×10^3 CFU/g, with a mean value of 5.4 Log CFU/g (Fig. 2). In such last case, as values over 10^3 CFU/g of CPS were detected, staphylococcal enterotoxins were also investigated according to Commission Regulation (EC) No 2073/2005 and their presence was always confirmed in 25 g (100%).

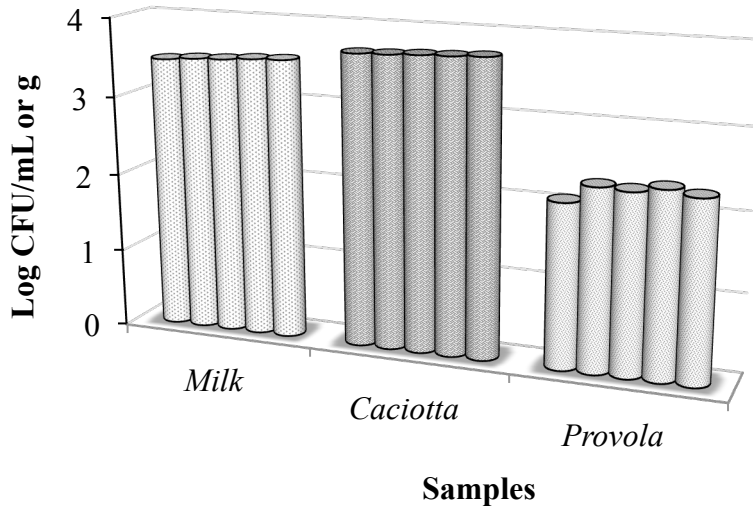


Figure 1. Coagulase positive staphylococci counts (CFU/mL or g) in milk and cheese samples.

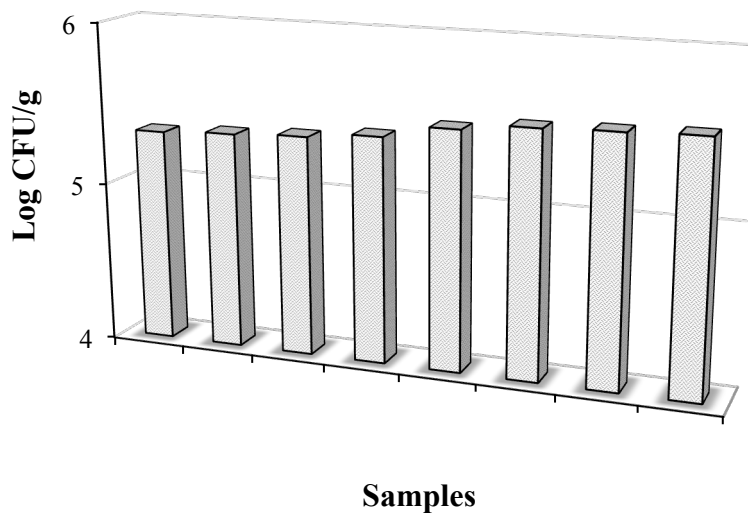


Figure 2. Coagulase positive staphylococci counts (CFU/g) in *mozzarella* samples exceeding the regulatory limit.

The results always below the detection limit for samples made in own-checks could be due to sampling made not at random, at least by some dairy industries – rather they aimed at selecting less manipulated products or those perceived to be safer, in order to reduce the possibility of encountering samples positive to CPS. On the contrary, some samples taken in the framework of official controls showed high values of CPS, stressing the importance of controls directly carried out by competent authorities. However, they usually did not exceed the process hygiene criteria, except for *provola* and *mozzarella* samples. These results could suggest the importance of good hygiene and manufacturing practices during cheese production in the first case, whereas the presence of staphylococcal enterotoxins in *mozzarella* samples with high values of CPS ($> 10^5$ CFU/g) confirmed a potential serious risk for consumers. All positive samples belonged to the same batch produced in a small

dairy industry in Molise region and were withdrawn or recalled in accordance with Article 19 of Regulation (EC) No. 178/2002 (EC, 2002). That batch was probably contaminated, even though it remains unknown if such contamination could be due to raw materials, lack of good hygiene practices or even to the handlers themselves, representing healthy carriers of CPS. However, some studies reported that pasteurized milk cheese caused outbreaks of foodborne diseases also at a higher incidence rate than those linked to raw milk cheese consumption (YOON *et al.*, 2016). Moreover, failure of thermal processing, contamination of the products after pasteurization or abuse of storage temperature could allow the proliferation of CPS in dairy products as well, after heat treatment. Some bad hygiene habits such as coughing or sneezing and not washing hands when handling cheese production equipment could be a source of contamination of the end products (ROSENGREN *et al.*, 2010).

Other authors (DELBES *et al.*, 2006) reported lower CPS values than our results in raw cow's milk collected from France, ranging from undetectable to about 1.1×10^3 CFU/mL. As far as cheeses are concerned, ROSENGREN *et al.* (2010) detected CPS in 69% of raw milk cheeses and 6% of cheeses made from pasteurized milk, even if staphylococcal enterotoxins were never detected. According to the results of our investigation, mean values of CPS (2.6×10^3 CFU/g) were detected in raw milk cheese produced in a South Tyrolean malga and the presence of staphylococcal enterotoxins was also confirmed, causing symptoms referable to staphylococcal food poisoning (ARMANI *et al.*, 2016). According to the European Union summary report on trends and sources of zoonoses in 2016 (EFSA and ECDC, 2017), a monitoring study on milk samples from different animal species carried out in Italy reported that 110 and 55 out of 165 samples were positive for *S. aureus* and *Staphylococcus* spp. respectively. Another Italian survey on 6,482 milk samples described in the above quoted report showed that 451 were positive for *S. aureus*, 54 for *Staphylococcus intermedius* and 628 for *Staphylococcus* spp. JOHLER *et al.* (2018) found 80% of artisan raw milk cheese samples positive to *S. aureus* from 25 dairies located in Italy. Some isolates exhibited at least one gene encoding some of staphylococcal enterotoxins. Another Italian study (CREMONESI *et al.*, 2007) on raw milk cheese samples (fresh, soft, semi-hard and hard cheeses) reported that no enterotoxins were detected in samples positive to high concentrations of *S. aureus*.

4. CONCLUSIONS

The results of the present study have highlighted the presence of CPS in some raw milk and unripened cheese, as well as the detection of staphylococcal enterotoxins in eight samples of *mozzarella*. Therefore, the monitoring of CPS incidence in milk and dairy products represents an important tool for public health, especially when high values are detected. The ubiquity of such microorganisms and the lack of good manufacturing practices in milk and cheese production represent a risk factor to consumers, so that the improvement of hygiene procedures such as regular checks of animal health, testing of milk and monitoring of critical points in the production process should be expected.

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