CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* ISOLATES FROM TRADITIONAL DAIRY PRODUCTS OF SMALL-SCALE ALPINE FARMS

V. FILIPELLO*[1], M. TILOLA[1], L. ZANI[2], B. BERTASI[1], M.V. LUINI[3] and G. FINAZZI[1]

[1]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via A. Bianchi 9, 25124 Brescia, Italy

[2]Department of Food and Drug, Università degli Studi di Parma, Parco Area delle Scienze 27/A, 47124 Parma, Italy

[3]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Sezione di Lodi, Via A. Einstein, 26900 Lodi, Italy

*E-mail address: virginia.filipello@izsler.it

ABSTRACT

This study investigated the prevalence of *Staphylococcus aureus* in raw milk dairy products handcrafted in traditional alpine small-scale farms, and characterised the enterotoxigenicity and resistance to methicillin. Among the analysed samples, 69% exceeded the international microbiological recommendations. The highest counts were observed for cheese or fatty products (~10^6 cfu/g). Conversely, lower contamination levels concerned raw milk and whey cheese (~10^2 cfu/g). A total of 163 *S. aureus* isolates were collected, and the prevalence of MRSA was low (1.7%) but not negligible. The finding of enterotoxins genes in 67% of the isolates is of concern for the public health.

Keywords: alpine small-scale dairies, dairy products, molecular characterisation, staphylococcal enterotoxins, *Staphylococcus aureus*

1. INTRODUCTION

Staphylococcal Food Poisoning (SFP) is one of the most common foodborne diseases worldwide caused by the ingestion of food contaminated with preformed Staphylococcal Enterotoxins (SEs) produced by Staphylococcus aureus (HENNEKINNE and DRAGACCI, 2012). SFP is generally characterised by self-limiting gastrointestinal symptoms, but occasionally the disease can be more severe or even fatal (BENKERROUM, 2017). *S. aureus* is ubiquitous in the environment and it is also one of the major causes of bovine mastitis (Boss *et al*., 2016). Therefore, raw milk and raw milk dairy products may be contaminated with *S. aureus*, due to the shedding of large segments of the organism into milk (D'AMICO and DoNnelly, 2011; ROLA and OSEK, 2016). Moreover, cheese-makers may carry enterotoxin-producing *S. aureus* in their noses or on their hands, and the lack of proper hygienic measures during food processing increases the probability of contamination with *S. aureus*, especially in small-scale artisanal dairies (ANDRÉ *et al*., 2008). Indeed, dairy products are among the foods most commonly involved in SFP outbreaks (BENKERROUM, 2017; DE BUYSER and LAFARGE, 2001).

To date, 23 different SEs have been described and many *S. aureus* strains harbour more than one SEs gene. SEs can be divided into classic types (i.e. A to E) and new variants classified at present as SEs or SEs-like (SEls) based on their ability to cause emesis. SEs are synthesised when *S. aureus* cell density reaches 10^5-10^8 cfu g^{-1}. However, all of these toxins are heat-stable and can therefore be still present in the food even when the microorganism is inactivate or the contamination level is reduced by processing (BENKERROUM, 2017).

Among *S. aureus* strains, those that are Methicillin-resistant (MRSA) have spread in the last decades as hospital-acquired pathogens (HA-MRSA) throughout the world, causing serious life-threatening infections not responding to a lot of antimicrobial treatments. More recently, community-acquired (CA-MRSA) and livestock-associated (LA-MRSA) MRSA have also emerged (BARDIAU *et al*., 2013). MRSA have been identified in different foods worldwide, and several food-borne MRSA outbreaks have been reported demonstrating the zoonotic risk of transmission to humans (DOULGERAKI and NYCHAS, 2017). The screening of *S. aureus* isolates from food of animal origin is therefore essential to estimate the MRSA emergence and the related zoonotic hazard (Bardiau *et al*., 2013).

In alpine regions, in particular in the Lombardy Region, raw milk dairy products are handcrafted in small-scale artisanal dairies built in pastures. These products and practices are closely linked to environmental, economic and tourist aspects, important for the safeguard and development of alpine culture and society (DELLA TORRE, 2017). In this context, traditional cheeses represent appealing products to the new trends of searching for natural and authentic foods, and many of them have been awarded with the Protected Designation of Origin (POD) label (Lombardia, 2014). However, since the hygienic conditions of traditional plants are very diverse, a specific surveillance plan for the safety of cheese produced in pastures has been developed (Italian Ministry of Health, 2017). Data on *S. aureus* isolates recovered from small-scale alpine dairies are however scarce.

The aims of this study were to investigate the prevalence of *S. aureus* and to characterise isolates from the production chain of artisanal raw milk dairy products. In particular, we tested the isolates for the presence of enterotoxins genes and for resistance to methicillin.

2. MATERIAL AND METHODS

2.1. Retrospective database analysis

IZSLER database was asked to obtain data on the prevalence and level of contamination of *S. aureus* for all milk and dairy products samples referred to our laboratory throughout 2016. Samples with $\geq 10^2$ cfu g^{-1} *S. aureus* counts were considered as exceeding international microbiological recommendations *(Reg. CE n. 2073/2005)*.

2.2. Sample selection and S. aureus isolation and identification

S. aureus isolates were collected from products tested within the alpine pastures surveillance plan carried out in the Lombardy Region. The samples were collected in 2016 from a total of 40 small-scale dairies. For *S. aureus* isolation, serial dilution of each sample homogenate were plated on Baird Parker agar + rabbit plasma fibrinogen (RPF agar) (Biolife Italiana, Milano, Italy) and incubated at 37°C for 48 h. Up to 5 characteristic colonies for each sample were planted on blood agar to confirm *S. aureus* hemolytic property. The species identification was confirmed with PCR of the *nuc* gene as described by Brakstad *et al*. (BRAKSTAD and MAELAND, 1992). DNA was obtained by boiling a suspension of the isolates in 2 ml of demineralised water for 5 min at 99°C. The suspension was then centrifuged at 13 000 g for 5 min and supernatant was used for all the following PCR assays.

2.3. Detection of mecA and mecC (methicillin resistance)

The detection of *mecA* and *mecC* (*mecA* homologue) was carried out by means of two PCR protocols using specific primers as reported by Pichon *et al*. (PICHON *et al*., 2012). Briefly, for both *mecA* and *mecC* the PCR reaction mix (final volume 20 µL) contained 1X HotStarTaq Master Mix (Qiagen INC, Hilden, Germany), 0.5 µM of each primer, and 1 µL DNA. The thermic profile was 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 1 min. The final elongation step was performed at 72°C for 10 min. The amplified PCR products were distinguished by electrophoresis in a 2.5% agarose gel (Agarose Multi Purpose, Roche -120 V for 40 minutes), stained with Eurosafe Nucleic Acid Stain (Euroclone, 1X). 100 bp DNA ladder (Invitrogen, 0.5 µg/µL) was included.

2.4. Staphylococcal enterotoxins

Two multiplex PCR protocols were used as described in Bianchi *et al*. (BIANCHI *et al*., 2014) to detect *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selj*, *selp*, and *ser* SEs genes. The electrophoresis conditions were the same for detection of mecA and mecC.

3. RESULTS AND DISCUSSION

3.1. Retrospective database analysis

Among the 4177 samples of milk or milk-derived products of different origin analysed by IZSLER for the presence of coagulase positive staphylococci during 2016, 145 were from small alpine pastures dairies. While for the other dairy products those exceeding the international microbiological recommendations were 22% (867/4032), for the traditional alpine products the proportion increased to 69% (100/145). The level of contamination varied between the different products tested. It is interesting to note that, in general, raw

milk has lower contamination values than the final products (Fig. 1). This could be due to an exponential growth of *S. aureus* in the early phases of cheese-making, when the milk is heated to about 40°C, which is the optimum temperature range for *S. aureus* growth and enterotoxin production (HENNEKINNE *et al*., 2012). In addition, secondary events of contamination from the cheese-maker's skin may happen due to inappropriate hygienic procedures. The only product with low level of contamination is whey cheese; as for its production, the whey is heated above 85°C.

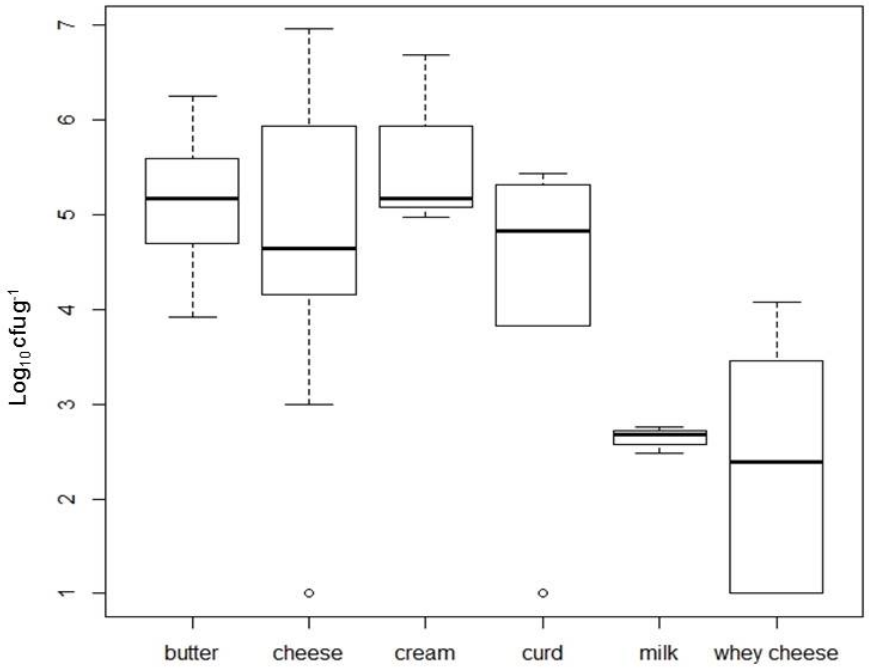

Figure 1. Distribution of the *S. aureus* counts in the different products analysed.

3.2. Isolates

Out of a total of 81 samples (n=23 from raw milk, n=7 from curd, n=39 goat or bovine cheese, n=11 butter, n=1 cream), 172 coagulase positive staphylococci isolates have been collected. A total of 163 (95%) isolates were confirmed as *S. aureus* by the *nuc* PCR, and used for further characterisation (S1).

3.3. MRSA isolates

Among the 163 isolates analysed, 3 (1.7%) were MRSA (*mecA*+; S1). None of the isolates was found to be *mecC* positive. The isolation frequency of MRSA raw milk and dairy products in the present study is consistent with the low prevalence estimates previously reported. Studies from Greece and Italy have revealed MRSA prevalence estimates of 3% (PAPADOPOULOS *et al*., 2018), 3.8% (CORTIMIGLIA *et al*., 2016) and 0.7% (GIACINTI *et al*., 2017). However, given the fact that traditional herding systems on alpine pastures should be extensive and characterized by low rates of antimicrobials administration, the results of this study raise some concern.

3.4. SEs genes detection

At least one SEs gene was found in 67% of the isolates (n=110) and 29 different SEs genes profiles were distinguished (Table 1; S1).

Table 1. Enterotoxins gene profiles. The number at the end of each line represents the number of isolates bearing a specific enterotoxins gene profile. The number at the bottom of each column represents the number of isolates bearing a specific enterotoxin gene.

Enterotoxins genes											
sea	**seb**	**sec**	**sed**	**see**	**ser**	**seg**	**seh**	**sei**	**selj**	**selp**	**no. of isolates**
•											21
•	•		•		•				•		1
•		•									4
•			•								4
•			•				•				2
•			•		•						5
•			•		•				•		13
•						•		•			2
•							•				1
•					•				•		4
•					•				•		1
	•										9
		•									4
		•				•		•			1
			•								9
			•			•		•			1
			•				•				1
			•						•		4
			•		•				•		10
			•		•				•	•	1
				•							1
						•					1
						•		•			2
							•				2
							•	•			1
								•			1
									•		1
					•						2
					•		•	•	•		1
58	10	9	51	1	38	7	8	9	36	1	

sea was detected in 53% (n=58) of the isolates, followed by *sed* (n=51; 46%), *ser* (n=38; 35%) and *selj* (n=36; 33%) genes. SEA and SED are the SEs most frequently associated with SFP, and they have caused outbreaks linked to the consumption of dairy products (HUMMERJOHANN and GRABER, 2014; JOHLER *et al*., 2015; SABIKE and EDRIS, 2014). Twenty-five isolates (23%) contained the SEs gene pattern *sed*, *sej*, *ser*, which are carried on

the same plasmid (BENKERROUM, 2017), with more than half of them (14/25) additionally carrying *sea*. These patterns have been correlated with genotype B *S. aureus* as identified by RS-PCR, which has been reported to be a particularly virulent bovine-associated type of *S. aureus*, and the one most widespread in Switzerland and central European countries (HUMMERJOHANN *et al*., 2014).
For SED and SER, the exhibition of emetic activity is well established (SCHUBERT and BANIA, 2017), while the situation for SElJ remains unclear (BENKERROUM, 2017). Nevertheless, all SEs and SEls belong to the family of superantigens, molecules able to stimulate T-cell proliferation (5000-fold more than in a conventional immune response), driving a massive release of cytokines that cause a life threatening systemic inflammation and toxic shock (TSS). However, to date it is not clear whether exposure to SEs/SEls via food can lead to TSS, and it has been suggested that it is the dose that makes the difference between evolution in TSS or SPF in case of SEs/SEls ingestion (BENKERROUM, 2017). *seh*, which also has been linked to milk-based SFP outbreaks (BIANCHI *et al*., 2014), was detected in 8 isolates (7%).
Improper handling and storage of raw milk and cheese in the early stages of processing contaminated with *S. aureus* can result in the production of SEs, which is also dependent on the initial dose of *S. aureus* contamination (SABIKE *et al*., 2014). Based on our data, the contamination of raw milk averaged 10^{2} cfu g^{-1}, while the average contamination of cheese was 10^{6} cfu g^{-1}, indicating that the alpine pasture process of cheese-making allows exponential growth of *S. aureus*, that reaches a concentration critical for the production of SEs (10^{5}-10^{8} cfu g^{-1};(BENKERROUM, 2017). Indeed, one of the samples included in our study was referred to our laboratory for the suspect involvement in a SFP episode. It was an aged cheese (isolate 20.1) which proved positive for SEA even if the count of *S. aureus* was 10^{3} cfu g^{-1} (data not shown) suggesting that the *S. aureus* population declined during aging. In the European Union, milk-derived products are examined for enterotoxin content only when the number of coagulase-positive staphylococci exceeds 10^{5} cfu g^{-1} (Reg. CE n. 2073/2005). In the light of our findings, this measure may not be appropriate with regard to aged cheese. Moreover commercial kits commonly used for SEs detection are only available for classical SEs (i.e. A to E), leading to an underestimation of the actual incidence of new SEs and SEls. Conversely, the production of SEs/SEls depends on the expression of the SEs/SEls genes, which is dependent on a complex regulatory system influenced by specific environmental conditions (i.e. temperature, pH, a_w, Eh, and salt concentration). It is therefore possible that even when the *S. aureus* contamination reaches critical levels, the SEs/SEls are not produced, highlighting again the modest value of *S. aureus* count as indicator of the presence or absence of SEs in food (BENKERROUM, 2017). Indeed, this situation is routinely observed in our laboratory (data not shown).

4. CONCLUSIONS

Despite the high overall *S. aureus* prevalence (69%) in dairy products manufactured in alpine small-scale farms, the estimated MRSA prevalence in our study was low (1.7%) but not negligible. It is therefore necessary to keep monitoring foods and apply control measures against *S. aureus* in herds to minimise the dissemination of MRSA in animals and subsequently in the community.
Milk and milk products are considered to be of particular significance as a staphylococcal enterotoxin (SE) source. Given the high levels of contamination found in many of the products analysed, the presence of enterotoxigenic strains of *S. aureus* should raise concern. Indeed, the technologies used in alpine pasture dairies are not effective in hindering and limiting the proliferation of *S. aureus* in the early phases of production, and

both human and animal sources can be responsible for contamination. Within this scope, in traditional dairies major benefits could derive from the application of basic good manufacturing practices, starting from control of the health status of cows and milking hygiene. Focused educational interventions and further studies aimed at assessing the routes of transmission of *S. aureus* in small-scale alpine farms could have a great impact on the quality and safety of these precious and peculiar productions.

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