Short communication: Characterization of *Staphylococcus aureus* isolated along the raw milk cheese production process in artisan dairies in Italy

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ABSTRACT

Staphylococcus aureus is a common cause of food-borne intoxications. Several staphylococcal food poisoning outbreaks have been linked to consumption of raw milk cheeses and artisanal cheese production. However, information on *Staph. aureus* isolated from artisanal raw milk cheeses and small-scale dairy production environments is very limited. Therefore, we aimed to characterize *Staph. aureus* isolated along the artisanal raw milk production chain by determining (1) the population structure, and (2) the presence/absence of enterotoxin genes, *mecA/C*, and *pvl*. We collected 276 samples from different production stages (raw milk, whey, curd, brine, drying worktops, and cheese) at 36 artisan dairies in Italy. A total of 102 samples from 25 dairies tested positive for *Staph. aureus*, with 80% positive samples among the tested artisan cheeses. All isolates were further characterized by *spa* typing and PCR screening for staphylococcal enterotoxin genes, the *mecA/mecC* genes characteristic for methicillin-resistant *Staph. aureus*, and the *pvl* gene encoding Panton-Valentine leukocidin. The 102 isolates represented 15 different *spa* types and were assigned to 32 different *Staph. aureus* strains. The *spa* type most frequently detected was t2953 (30%), which is associated with genotype B strains causing high within-herd levels of bovine mastitis. In addition, 3 novel *spa* types (t13269, t13277, and t13278) were identified. Although none of the strains harbored *mecA/mecC* or *pvl*, 55% of the isolates exhibited at least one enterotoxin gene. Many strains were present in samples from multiple dairies from different regions and years, highlighting the spread of *Staph. aureus* in small-scale cheese production plants. Our findings demonstrate that enterotoxigenic *Staph. aureus* and in particular t2953 (genotype B)

isolates commonly occur in artisanal dairies and raw milk cheeses in Italy. It is particularly alarming that 80% of the artisan cheeses sampled in our study were positive for *Staph. aureus*. These findings stress the need for effective measures preventing staphylococcal food poisoning by limiting *Staph. aureus* growth and enterotoxin formation along the production chain and in the final product.

Key words: *spa* typing, enterotoxin, staphylococcal food poisoning, methicillin-resistant *Staphylococcus aureus*

Short Communication

Staphylococcus aureus is frequently isolated from raw milk intended for cheese production, dairy processing equipment, and environments, as well as food handlers. Its presence in raw milk represents a source for introduction of *Staph. aureus* into the dairy product supply chain (D'amico and Donnelly, 2011). Ingestion of staphylococcal enterotoxins produced by *Staph. aureus* in food can result in staphylococcal food poisoning, and several outbreaks linked to consumption of raw milk cheeses and artisanal cheeses have been reported (Johler et al., 2015a,b). Enterotoxigenic strains originating from small-scale cheese productions may also carry a wide variety of important resistance and virulence determinants including *mecA/mecC*, conferring methicillin resistance or *pvl* coding for the β -pore-forming toxin Panton-Valentine leukocidin (Herrera et al., 2016).

Information on *Staph. aureus* from artisanal raw milk cheeses and dairy production environments is scarce. Although a large amount of the cow milk produced in Italy is used in the manufacture of typical raw milk cheeses, few data on the occurrence and genomic characteristics of *Staph. aureus* from these sources are available. A previous study screening raw milk and raw milk cheeses in the Turin area reported that 39% of samples were positive for *Staph. aureus* and that 21% of the isolates carried enterotoxin genes (Bianchi et al., 2014). Another study performed in the Apulia and Basilicata region detected methicillin-resistant *Staph. aureus* in

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2.5% of bulk tank milk samples tested (Parisi et al., 2016).

Phenotypic or genotypic typing methods can be used to discriminate different *Staph. aureus* isolates, for host attribution or to determine the relatedness of different isolates. Sequencing and analysis of the polymorphic X region of the protein A gene (*spa* typing) was shown to yield excellent discriminatory power (Koreen et al., 2004).

The aim of this study was to characterize isolates of *Staph. aureus* from raw milk cheese productions of small-scale artisan cheese manufactures in Italy by determining *spa* types and the presence/absence of enterotoxin genes, *mecA/C*, and *pvl*.

All isolates used in this study were collected in Italy between 2010 and 2012. We sampled and re-sampled 36 dairies producing artisanal raw milk cheeses from bovine milk in Piedmont and Aosta Valley. Samples were taken from raw milk (n = 36), whey (n = 36), curd (n = 36), brine (n = 36), drying worktops (n = 36), and cheese (n = 96). Information on sample type, dairy, and year is included in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13815>). Food samples were processed according to the ISO 6888–2 (ISO, 1999) method for the detection and enumeration of coagulase-positive staphylococci. For each sample, 5 colonies displaying a phenotype characteristic for coagulase-positive staphylococci were streaked on blood agar plates and identified as *Staph. aureus* using the VITEK system GP card (bioMérieux, Marcy l'Etoile,

France). Environmental swab samples were placed in 10 mL of Mueller-Hinton broth (Oxoid, Basingstoke, UK) supplemented with 6.5% NaCl (Sigma-Aldrich, Arklow, Ireland), vortexed, and incubated for 24 h at 37°C. After incubation, an aliquot of 100 µL was pour plated in rabbit plasma fibrinogen Baird Parker agar plates (Oxoid) and incubated at 37°C. Species confirmation for colonies displaying a *Staph. aureus* characteristic phenotype after 24 or 48 h was performed by using a VITEK system GP card (bioMérieux).

Genomic DNA was extracted from *Staph. aureus* strains using InstaGene Matrix (Bio-Rad, Milan, Italy). Each colony was suspended in 100 µL of matrix buffer, incubated at 56°C for 60 min, followed by incubation at 95°C for 45 min. The suspensions were centrifuged at 20,000 × g for 5 min and supernatants were used for PCR screening of all isolates for enterotoxin genes, *pvl*, *mecA*, and *mecC*. Enterotoxin genes were detected using 2 multiplex PCR assays (see Table 1) according to the protocols of the European Union Reference Laboratory for Coagulase-Positive Staphylococci (Kérouanton et al., 2007). The following reference strains were included as positive controls: FRIS6 (*sea*, *seb*), FRI137 (*seg*, *seh*, *sei*), FRI326 (*see*), FRI361 (*sec*, *sed*, *ser*), and HMPL280 (*seg*, *sei*, *selj*, *selp*). The PCR screening for *mecA/mecC* and *pvl* was performed as previously described (Stegger et al., 2012). We determined *spa* types as previously described (Johler et al., 2011) and depicted results by constructing a minimum spanning tree (BioNumerics software ver. 7.6, Applied Maths Inc., Austin, TX). Iso-

Table 1. Primers used for the detection of *Staphylococcus aureus* enterotoxin genes by a multiplex PCR approach¹

Gene	Primer	Primer sequence (5'–3')	Product size (bp)	Reference
<i>sea</i>	GSEAR-1	GGT TAT CAA TGT GCG GGT GG	102	Mehrotra et al., 2000
	GSEAR-2	CGG CAC TTT TTT CTC TTC GG		
<i>seb</i>	GSEBR-1	GTA TGG TGG TGT AAC TGA GC	164	Mehrotra et al., 2000
	GSEBR-2	CCA AAT AGT GAC GAG TTA GG		
<i>sec</i>	GSECR-1	AGA TGA AGT AGT TGA TGT GTA TGG	451	Mehrotra et al., 2000
	GSECR-2	CAC ACT TTT AGA ATC AAC CG		
<i>sed</i>	GSEDR-1	CCA ATA ATA GGA GAA AAT AAA AG	278	Mehrotra et al., 2000
	GSEDR-2	ATT GGT ATT TTT TTT CGT TC		
<i>see</i>	SA-U	TGT ATG TAT GGA GGT GTA AC	213	Sharma et al., 2000
	SA-E rev	GCC AAA GCT GTC TGA G		
<i>ser</i>	SER 1	AGA TGT GTT TGG AAT ACC CTA T	123	Chiang et al., 2008
	SER 2	CTA TCA GCT GTG GAG TGC AT		
<i>seg</i>	SEG-F	GTT AGA GGA GGT TTT ATG	198	Bania et al., 2006
	SEG-R	TTC CTT CAA CAG GTG GAG A		
<i>seh</i>	SEH-F	CAA CTG CTG ATT TAG CTC AG	173	Bania et al., 2006
	SEH-R	CCC AAA CAT TAG CAC CA		
<i>sei</i>	SEI-F	GCG CAC TTT ATC AGG ACA	328	Bania et al., 2006
	SEI-R	AAC TTA CAG GCA GTC CA		
<i>selj</i>	SEJ-F	GTT CTG GTG GTA AAC CA	131	Bania et al., 2006
	SEJ-R	GCG GAA CAA CAG TTC TGA		
<i>selp</i>	SEP-F	TCA AAA GAC ACC GCC AA	396	Bania et al., 2006
	SEP-R	ATT GTC CTT GAG CAC CA		

¹Annealing temperatures used were 55°C for the first multiplex PCR (*sea-see*, *ser*), and 52°C for the second multiplex PCR (*seg-selj*, *selp*).

lates with identical *spa* types, as well as virulence and resistance gene profiles, were considered to represent the same strain.

Out of 276 samples from 36 dairies, 102 samples from 25 dairies tested positive for *Staph. aureus*. Positive samples were detected along the whole production chain (Table 2), with 19% positive raw milk samples, 6% positive whey samples, 14% positive curd samples, 11% positive brine samples, and 19% positive samples from drying worktops. Eighty percent of the samples originating from the artisan raw milk cheese itself were positive for *Staph. aureus*. This is consistent with a previous study (Johler et al., 2016) showing that the organism can enter the artisanal cheese-making process at different stages of the manual production, with colonized cheese makers representing a likely source of *Staph. aureus*. A total of 55% of all isolates and 56% of the cheese isolates exhibited at least one gene encoding SEA, SEB, SED, SEIJ, SEG, SEI, or SER. Most enterotoxigenic isolates harbored both classical (*sea-see*) and new staphylococcal enterotoxin genes (*ser*, *seg*, *seh*, *sei*, *selj*, *selp*). Consistent with the findings of Blaiotta et al. (2006), *seg* and *sei* always coexisted and were never detected independently of the other gene. A previous study screening Italian raw milk and raw milk cheeses reported that 39% of samples were positive for *Staph. aureus*, with 21% of the isolates carrying enterotoxin genes (Bianchi et al., 2014). No Pantone-Valentine leukocidin or methicillin resistance genes were detected, consistent with previous studies demonstrating low prevalence of these genes among *Staph. aureus* isolated from dairy products in Italy (Spanu et al., 2012; Mancini et al., 2015; Parisi et al., 2016). A complete list of all *Staph. aureus* isolates detailing the dairy, sample type, year of isolation, *spa* type, and repeat sequence, as well as the results of the PCR screening for enterotoxin and virulence (*mecA/C*, *pvl*) genes, is provided in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-13815>).

The 102 isolates were assigned to 15 different *spa* types. The most common *spa* types were t2953 (*n* = 30), t524 (*n* = 14), and t267 (*n* = 11). In a previous

study, t2953 was one of several *spa* types associated with *Staph. aureus* strains of genotype B, which are exclusively linked to high within-herd prevalence of bovine mastitis (Graber et al., 2009; Boss et al., 2016). *Staphylococcus aureus* of genotype B were reported to represent a major contaminant in raw milk cheeses in Switzerland and were identified as the causative agent in outbreaks of staphylococcal food poisoning (Humerjohann et al., 2014; Johler et al., 2015b). *Staphylococcus aureus* of *spa* type t524 have been commonly detected in bovine mastitis milk in various countries (Hasman et al., 2010; Johler et al., 2011; Sakwinska et al., 2011). *Staphylococcus aureus* t267 strains have been isolated from mastitis milk in cattle and sheep (Johler et al., 2011; Merz et al., 2016), from poultry (Krupa et al., 2014), and from samples of human patients suffering from bloodstream infections (Nulens et al., 2008). In addition, the first reported vancomycin-resistant *Staph. aureus* detected in bovine milk, which was isolated in India, belonged to t267 (Bhattacharyya et al., 2016). We constructed a minimum spanning tree to depict the frequency and genetic relatedness among *spa* types detected in this study (Figure 1). Three novel *spa* types were identified: t13269 (*n* = 3), t13277 (*n* = 5), and t13278 (*n* = 2). Each of these *spa* types was detected in samples from 2 different dairies. The *spa* types t13278 (repeat succession: 11–12–12–22–25) and t13269 (repeat succession: 11–12–12–22–25–25) are closely related and differ only by one *spa* repeat. A comprehensive list of all *spa* types detected, as well as associated dairies and virulence and resistance genes, is included in Table 3.

The 102 isolates collected in this study represented 32 strains, 10 of which were isolated from more than one dairy. The enterotoxigenic genotype B strain S22 (t2953; *sed/selj/ser*) was isolated from 21 samples originating from raw milk, brine, a drying worktop, and cheese samples collected over a time span of 3 yr from 10 different dairies. In approximately two-thirds of the dairies (69%), at least one *Staph. aureus* strain was detected. Although 47% harbored only one strain, 22% of the dairies were linked to more than one *Staph. aureus*

Table 2. *Staphylococcus aureus* isolates detected in 25 out of 36 tested artisan dairies along the cheese production process

Production stage	No. of samples taken	No. of isolates detected	Prevalence (%)
Raw milk	36	7	19
Whey	36	2	6
Curd	36	5	14
Brine	36	4	11
Drying worktops	36	7	19
Raw milk cheese	96	77	80
Total	276	102	37

Figure 1. Minimum spanning tree visualizing the *spa* typing results. Each *spa* type is depicted by a single node, with the size of the node representing the number of isolates associated with this *spa* type. Color version available online.

strain. The most extreme case was dairy D1, which harbored 15 different *Staph. aureus* strains identified in samples from brine, curd, drying worktop, and cheese samples. Adaptation of individual production processes to inhibit *Staph. aureus* growth and enterotoxin formation (Cretenet et al., 2011; Johler et al., 2016) could reduce the risk of infection for the consumer.

Our findings show that enterotoxigenic *Staph. aureus* strains are very common all along the artisan raw milk cheese production process. As staphylococcal food poisoning outbreaks linked to raw milk cheeses and artisanal cheeses have been reported, the presented data on the occurrence and enterotoxigenicity of these strains are crucial to improve risk assessment and to determine the spread and distribution of isolates. It is particularly alarming that 80% of the samples taken from the final product were positive for *Staph. aureus*.

As these cheeses are predominantly intended for direct consumption, the high prevalence of *Staph. aureus* in artisan raw milk cheeses raises concern for human health. Stringent control measures are needed to inhibit growth and enterotoxin formation of *Staph. aureus* and to prevent staphylococcal food poisoning.

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Table 3. Overview of typing data and genotypes detected¹

<i>spa</i> type	Prevalence ² (%)	Strain (S)	Dairy (D)	Sample type	Enterotoxin genes
t024	1	S1	D1	Brine	<i>sed, selj, ser</i>
t164	5	S2	D1, D5	Cheese	<i>seg, sei</i>
		S3	D6	Raw milk, cheese	<i>sed, seg, sei, selj, ser</i>
t267	11	S4	D2, D4, D5, D6	Curd, brine, drying worktop	neg
		S5	D4	Cheese	<i>sed, ser</i>
t359	9	S6	D8, D10, D16, D17, D18	Raw milk, drying worktop, cheese	neg
		S7	D7	Cheese	<i>sed</i>
t442	3	S8	D1	Drying worktop	<i>sed, selj, ser</i>
		S9	D6	Cheese	<i>sed, seg, sei, selj, ser</i>
t521	7	S10	D1, D3, D15, D22	Whey, cheese	neg
		S11	D21	Cheese	<i>sed, selj, ser</i>
t524	14	S12	D1	Raw milk, drying worktop, cheese	neg
		S13	D1	Cheese	<i>sea</i>
		S14	D1	Cheese	<i>sea, ser</i>
		S15	D1	Cheese	<i>seg, sei</i>
		S16	D1	Drying worktop	<i>sed, selj, ser</i>
t529	4	S17	D7	Milk whey, cheese	<i>seg, sei</i>
t2953	30	S18	D1, D5	Cheese	neg
		S19	D24	Curd, cheese	<i>sed, selj</i>
		S20	D1	Cheese	<i>seg, sei</i>
		S21	D1	Curd, cheese	<i>sea, sed, selj</i>
		S22	D1, D2, D3, D4, D5, D7, D12, D13, D19, D20	Raw milk, brine, drying worktop, cheese	<i>sed, selj, ser</i>
		S23	D1	Cheese	<i>sea, sed, selj, ser</i>
t3802	5	S24	D5	Cheese	<i>sed</i>
		S25	D5	Cheese	<i>selj</i>
		S26	D5	Cheese	<i>sed, selj, ser</i>
t3987	1	S27	D25	Cheese	neg
t5268	1	S28	D1	Cheese	<i>selj, ser</i>
t13269	3	S29	D11	Raw milk, cheese	neg
		S30	D8	Curd	<i>sed, selj, ser</i>
t13277	5	S31	D3, D7	Cheese	neg
t13278	2	S32	D9, D14	Cheese	neg

¹None of the tested isolates harbored *mecA/mecC* genes characteristic of methicillin-resistant *Staphylococcus aureus* or the *pvl* gene encoding Pantone-Valentine leukocidin. neg = negative.

²Proportion of detected *Staphylococcus aureus* strains positive for the respective *spa* type.

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