The prevalence, genetic diversity and antibiotic resistance of *Staphylococcus aureus* in milk, whey, and cheese from artisan farm dairies

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Abstract

In this study, coagulase positive staphylococci (CPS) were detected in 45% of the 69 bovine milk, whey and cheese samples from five farm dairies, and all raw milk samples were contaminated. Genetic diversity, staphylococcal enterotoxin genes and antimicrobial susceptibility in putative *Staphylococcus aureus* isolates were investigated. Sixty-one percent of the 72 isolates analysed belonged to the same PFGE group. The *spa*-typing revealed seven different *spa* types, t2678 being the most prevalent, but t127 and t197 were also detected. Sixteen different toxin gene profiles were identified in 87.5% of the isolates with *sec* and *tst* being the most frequent (52.5%), followed by *seg* and *seh*. All isolates were MSSA (methicillin-sensitive *S. aureus*), and sensitive to the 12 antibiotics tested. The prevalence of *S. aureus*, and the high diversity of isolates carrying enterotoxin genes, constitute grounds for food safety concern in artisanal cheese making, whether pasteurized or not.

1 Introduction

Coagulase-positive staphylococci (CPS) are associated with bovine mastitis (Capurro, Aspán, Unnerstad, Waller, & Artursson, 2010; Jørgensen, Mørk, & Rørvik, 2005b; Verraes, et al., 2015) and can be transmitted into the dairy food chain, leading to contaminated dairy products, and possible staphylococcal food poisoning (SFP). The prevalence of *S. aureus*-positive bulk milk samples varies, with a reported range from 51% to 91% (Jørgensen, Mørk, Høgåsen, & Rørvik, 2005c; Katholm, Bennedsgaard, Koskinen, & Rattenborg, 2012; Walcher, et al., 2014). Furthermore, *S. aureus* is ubiquitous: detected in animals, cowsheds, farm and dairy workers, processing environments and ultimately the cheese (Jørgensen, et al., 2005b; Lim, et al., 2013). The pasteurization of raw milk for cheese production at an adequate temperature and time-period usually kills all vegetative bacteria present, but also those beneficial to the richness in taste and flavor and other associated advantages (Montel, et al., 2014). The screening and sampling of raw milk cheeses from farm dairies, local markets, specialty shops and on-line resources indicates a contamination level of *S. aureus* between 10% and 69% (Brooks, et al., 2012; Jakobsen, Heggebø, Sunde, & Skjervheim, 2011; Rosengren, Fabricius, Guss, Sylvén, & Lindqvist, 2010).

A variety of dairy products, e.g., mashed potatoes with raw milk (Jørgensen, et al., 2005a), low fat milk (Asao, et al., 2003), ice-cream (Fetsch, et al., 2014), and soft raw milk cheese (Johler, et al., 2015) have been associated with disease outbreaks. Intoxication is caused by staphylococcal enterotoxins (SE) pre-produced by *S. aureus* in the dairy product, and causes severe diarrhea and vomiting only hours after ingestion (Argudín, Mendoza, & Rodicio, 2010). The five major classical staphylococcal enterotoxins (SE) (SEA to SEE) are included in the 22 SEs, SEls (SE-like), and TSST-1 (Toxic Shock Syndrome Toxin 1) described so far and are thoroughly reviewed (Cretenet, Even, & LeLoir, 2011; Gustafson, Muthaiyan, Dupre, & Ricke, 2014; Hennekinne, De Buyser, & Dragacci, 2012). *S. aureus* SEs are found in a

wide diversity in a large proportion of dairy products (Carfora, et al., 2015; Rosengren, et al., 2010). The SE- and SEl-genes are primarily located on different types of mobile genetic elements, and the distribution of these elements can modify the pathogen and thereby contribute to its evolution (Alibayov, Zdenkova, Sykorova, & Demnerova, 2014). Multiresistant *S. aureus* emerged decades ago because of the widespread and often inappropriate use of antibiotics in livestock. Despite restrictions of use, both clinically and in food production, the trend of rising antibiotic resistance continues (EFSA, 2009; NSCFS, 2015). Methicillin resistant *S. aureus* (MRSA) is found in pigs, and in areas with a high livestock density of both cows and pigs. The probability of transferring MRSA to cows, e.g., through humans, and thereby to milk and cheese, is high (Locatelli, et al., 2016). Cheeses, particularly those made from raw milk, have been reported to contain high loads of resistant bacteria (Flórez, et al., 2014).

The classical approach to the analysis and understanding of the *S. aureus* population structure has been the relatively time-consuming and labor-intensive Pulse Field Gel Electrophoresis (PFGE) (Strommenger, et al., 2006; Vautor, Abadie, Guibert, Huard, & Pépin, 2003). Sequence based methods, such as *spa*-typing (sequencing of the X region of the protein A) (Koreen, et al., 2004) and multiple locus sequence typing (MLST) (Roussel, et al., 2015; Strommenger, et al., 2006), are frequently used. The advantages of sequencing are obvious; foremost is the comparability between laboratories worldwide, reproducibility of results, and ease of use. On *S. aureus* isolates from bovine mastitis, PFGE could better discriminate between the isolates than MLST and *spa*-typing (Ikawaty, et al., 2009); however, in a global study on isolates from human disease, bovine, and ovine mastitis, *spa*-typing performed best of the two (Koreen, et al., 2004).

Numerous studies have been performed on the quality of bovine bulk milk, and on the prevalence of pathogens in cheese, especially in raw milk cheese. This study presents a holistic approach by sampling CPS and *S. aureus* at different stages through the cheese making process and assessing the results and consequences through (i) the quantification of the CPS contamination, (ii) the assessment of the genetic relation and possible proportion of antibiotic resistance in the *S. aureus* population, and (iii) the detection of SE-genes. The possible risk for SFP from products from artisan cheese farm dairies is discussed.

2 Materials and methods

2.1 Sampling

Ten farm dairies producing cheese from bovine milk in the region of Mid-Norway were invited to participate in the project. The participating farm dairies were asked to provide the following samples: raw milk, cheese milk (pasteurized if applicable), whey, and two mature cheeses, from three different production batches of the same cheese type. One of the mature cheeses was stored at 4°C until the expiry date and then analysed. The term "mature cheese" represents cheese ready for the consumer market as decided upon by the producer. The dairies were asked to submit additional information about the farm, the dairy, and the production in a questionnaire. Supplementary information was collected through the respective county administrations. The producers were supplied with all the necessary equipment and instructions for sampling. The milk and whey samples were packaged in insulated polystyrene boxes with refrigerant gel packs and returned within two days. The box temperature was registered on arrival. The temperature in the milk and whey samples varied between 0°C and 4°C, with a mean value of 1.6°C, and the cheese samples had a mean value of 3.2°C (range

95 2°C to 6°C). The samples were stored at 4°C until analysis the next day. The study was
96 conducted during a five-month winter period.

97 **2.2 Quantification of CPS in milk, whey and cheese; isolation and identification of**

98 ***S. aureus***

99 The samples of milk and whey were mixed gently by inverting the sample tubes, and then ten-
100 fold dilutions of the milk were made using sterile peptone water (1.0 g bacteriological peptone
101 and 8.5 g/L NaCl). The cheese samples were prepared by homogenizing 10 g of cheese and
102 90 g of sterile peptone water for 1 min in Stomacher bags (Seward Medical, Norfolk, UK).
103 Additional ten-fold dilutions were made by using sterile peptone water. The appropriate
104 dilutions were spread on Baird-Parker (BP) agar (Oxoid) for the quantification of coagulase-
105 positive staphylococci (CPS) according to NMKL method no. 66 (NMKL, 2009). The plates
106 were incubated at 37°C for 48 hours. Up to five typical colonies from each sample were
107 isolated: black or grey, shining, and convex with a diameter of 1.0 to 1.5 mm after 24 hours,
108 and often surrounded by a clear zone. They were further propagated to pure cultures on Brain
109 Heart Infusion Agar (BHIA) (Oxoid), and preserved in BHI containing 20% glycerol at 80°C.
110 All the isolates were Gram-stained (Pro-lab Diagnostics) and tested for catalase activity and
111 coagulase production. Additionally, coagulase positive isolates were streaked on p-agar
112 (Roberson, Fox, Hancock, & Besser, 1992) supplemented with 7 mg/L of acriflavin (Sigma).
113 Growth in the full length of the streak was considered to be a positive reaction. Isolates with
114 all positive reactions described above were considered to be *S. aureus.* The detection limits of
115 CPS in the milk and whey samples are 10 CFU/ml, and 100 CFU/g in the cheese samples.

116 **2.3 Genetic analyses of *S. aureus* isolates**

The presumptive *S. aureus* - isolates (n=72) were genetically characterized using pulsed-field gel electrophoresis (PFGE) using SmaI as the restriction enzyme (Murchan, et al., 2003). Differentiation of the banding patterns was performed using Bionumerics (version 6.6; Applied Maths, Kortrijk, Belgium), and the *S. aureus* strain CCUG 41582 was used as a reference strain for the normalization of fingerprint data. DNA fragments less than 45 bp were excluded from fingerprint analysis. Each unique banding pattern was assigned to a pulsotype (PT), and a cluster analysis was performed. One representative isolate from each PT was used to create a dendrogram. The cluster analysis was performed using the Dice similarity coefficient with 1.0% optimising settings, and the dendrograms were created using the unweighted pair group method with arithmetic averages (UPGMA) with 1.0% tolerance. The cluster cut-off for the grouping of isolates was set to 70% (Rosengren, et al., 2010). One to eight isolates from the same PT were selected for further analyses for the presence of virulence genes and the identification of *spa* type.

The DNA was isolated from the pure cultures using an Easy-DNA™ Kit (Invitrogen) with preliminary lysozyme treatment. One colony of each isolate was grown overnight at 37°C in 5 ml of BHI. Overnight cultures (1.5 ml) were pelleted at 13000 rpm for 5 minutes, followed by washing the pellets with a Tris EDTA -buffer (1ml). The cells were lysed with a 150 µl lysozyme solution (5 mg/ml in a TE-buffer) at 37°C for 40 minutes. The DNA was quantified using PowerWave XS (BioTek), and a Take 3 plate with a TE-buffer as a blank. Confirmation of the *S. aureus*-isolates was performed by the detection of the *nuc* gene (Brakstad, Aasbakk, & Maeland, 1992). The distribution of enterotoxin genes was analysed using PCR, as previously described (Lovseth, Loncarevic, & Berdal, 2004; Monday & Bohach, 1999). However, the detection of enterotoxin genes was performed in a singleplex, and with a hybridization temperature of 58°C. The amplification products were detected on a 1.5%

agarose (Seakem) gel. Detection of the *blaZ* and *mecA* genes was performed as described earlier (Kaase, et al., 2008; Murakami, et al., 1991).

Selected isolates for each PT were further characterized by the sequencing of the protein A gene. The X region of the *spa* gene was amplified by PCR as earlier reported (Harmsen, et al., 2003). The DNA sequences were obtained with an ABI 377 sequencer (Applied Biosystems, Foster City, Calif.). The *spa* types were determined, based on the sequencing results, using the Spatype PlugIn included in the Bionumerics (Applied Maths; version 6.6) software. The *spa* server (spa.ridom.de/spatypes) was used as well to predict the sequence types (STs).

2.4 Antimicrobial susceptibility testing

Selected isolates (n=30) were tested for their susceptibility to a panel of 12 antibiotics using the disc diffusion method following the guidelines from EUCAST (European Committee on Antimicrobial Susceptibility Testing, www.eucast.org). The panel consisted of cefoxitin (30µg), erythromycine (15 µg), clindamycin (2 µg), fucidin (10 µg), linezolid (10 µg), trimethoprim-sulfamethoxazole (1.25-23.75 µg), tetracycline (30 µg), ceftaroline (5 µg), gentamicin (10 µg), mupirocin (200 µg), norfloxacin (10 µg), and rifampicin (5 µg) (all Oxoid). The isolates were classified as susceptible or resistant, based on the *S. aureus* epidemiological cut-off values issued by the EUCAST.

2.5 Statistical analysis

To compare the mean levels of CPS in different groups of milk and cheese samples, a one-way ANOVA, followed by Tukey's HSD test, were performed on log-transformed data using the statistical software SPSS Statistics (Version 22, IBM). A significance level of 0.05 was used, and a bacterial counts equal to zero was scored as 1 CFU/g or ml.

3 Results

3.1 The farm dairies

Five of the ten invited farm dairies participated in the study. These farms were located in Mid-Norway, a region covering 56 km^3 of untouched nature interspersed with villages and a couple of cities. Farms A and E (Table 1) are located less than 100 km from each other, as are farms B and D. The distance between these two localities is more than 270 km, and farm C is more than 270 km from all the others. The participating farms differed both in their milk treatment before cheese making and the types of cheeses produced (Table 1). Two of the farm dairies (A and B) did not pasteurize the cheese milk, while the other dairies (C, D and E) pasteurized their cheese milk in the vat, and submitted milk samples from before and after pasteurization, accordingly (Table 1). Farms A, D and E produced soft cheeses while farms B and C produced semi-hard cheeses (Table 1). All of the farm dairies used a starter culture in their cheese production.

Table 1

3.2 CPS in milk, whey and cheese

Table 2

The mean level of CPS in the raw milk from the different farm dairies varied between 1.7 log CFU/ml and 2.7 log CFU/ml (Table 2A). CPS were detected in 45% of the 69 samples of milk, whey and cheese collected from the farm dairies. From each farm dairy CPS were present in 42% (A), 92% (B), 27% (C), 40% (D), and 33% (E) of the samples. The prevalence of CPS in the raw milk samples from pasteurized and non-pasteurized cheese production was 100%, with a mean level of 2.3 log CFU/ml (a range of 1.5 to 3.0 log CFU/ml) (Table 2B). Pasteurization significantly ($p < 0.05$) reduced the mean level of CPS in milk for cheese-making. However, sporadic colonies of CPS were detected after pasteurization in whey and

188 cheese samples from farms C, D, and E, but no CPS were detected in cheese at the expiry
189 dates from these farms (Table 2B). The mean level of CPS in cheese made from non-
190 pasteurized milk was not significantly lower than that in raw milk. Throughout the cheese
191 making process, CPS were only detected at farm B, which was producing semi-hard cheese
192 with levels between 2.2 log CFU/ml or /g to 3.1 CFU/ml or /g (Table 2A).

193 **3.3 Genetic analyses isolates from *S. aureus* in raw milk, whey, and farm cheese**

194 **Figure 1**

195 From 69 samples of milk, whey, or cheese, presumptive *S. aureus*-isolates (n=72) were
196 identified. Most of the isolates were from raw milk (66%), while 24 % and 10% were from
197 whey and cheese, respectively. Macro-restriction with SmaI produced 9 to 16 fragments, with
198 an overall relatedness of 36.5% (Figure 1). Three of the isolates were not able to be typed
199 using this method. Using a cut-off value of 70%, the patterns could be divided into 13 groups
200 or pulsotypes (PT). The dominant group consisted of 4 closely related PTs (07.1- 07.4)
201 containing the majority (61%) of the isolates. These closely related PTs were found in
202 samples from all of the farm dairies (A, B, C, D, E). Moreover, *S. aureus* isolates with a
203 different PT could be detected from the same farm dairy (Figure 1, farm B) and from the same
204 raw milk sample (Figure 1: isolates 227 and 232). Seven different *spa* types were detected
205 (Figure 1). The most frequent *spa* type found was t2678. This *spa* type was identified in
206 several PTs, and the *spa* types t346, t544, and t2678 were identified in isolates with the same
207 PT from the dominant group (Figure 1, PT 07.3). The *spa* types t544, t2678, and t3495 differ
208 only in the number of one of their repeats, are closely related, and probably belong to the
209 same lineage: CC133. The *spa* types t127 and t197 belong to ST1 and ST94, respectively.

210 **Table 3**

The selected presumptive *S. aureus*-isolates (n=72) contained the *nuc* gene confirming *S. aureus*. Further characterization revealed 16 different toxin gene profiles, and SE-genes were detected in 87.5% of the analysed *S. aureus* isolates (Table 3). The majority of the isolates (65%) had more than one enterotoxin gene. The most frequent SE-genes were *sec* and *tst*, in different combinations, in 52.5% of the isolates, followed by *seg* (35%) and *seh* (30%). The *sea* and *seb* were only detected in one isolate each, and in combination with other toxin genes (Table 3). The largest diversity in toxin gene profiles was found at farms B and D, with 16 and 12 profiles, respectively. Sixty percent of the isolates with enterotoxin gene profiles (II-VIII, X, XIV-XVI) were detected in raw milk, and all five dairies were represented. In whey, 5 enterotoxin gene profiles (IV, V, IX, XI, XII) were detected in 17.5% of the isolates from farms B and D. In cheese, mature and at expiry date, 10% of the isolates with 3 different profiles (III, XII, XIII) were also observed from farms B and D. Isolates from farm B were found at every sample point throughout the whole cheese making process. All of the isolates were characterized as MSSA (methicillin susceptible *S aureus*). The *blaZ* gene was identified in 6.5% of the isolates. All of the isolates were susceptible to the antibiotics included in this study (data not shown).

4 Discussion

Farm products are popular and in demand, and is distributed through farmer's markets, retail, and foodservice establishments. Farm dairies are mainly run by family members, all of whom participate in all aspects of the work, whether taking care of the animals or producing the cheeses. Rosengren et al. (2010) points out that a situation like this calls for effective hygiene barriers. That a food production premise is located close to farm animals, and all that that entails (faeces, manure, rodents, insects, etc.), emphasizes this point. In addition, cheese making is a challenging production, where food safety is relying on a close monitoring of the

process and strict hygiene practices. Food safety knowledge in local cheese production is more important than ever.

In the present study, all of the raw milk samples contained CPS. Most studies on CPS in raw milk from the last decade reported a prevalence ranging from 47.2% to 94.3% (D'Amico & Donnelly, 2010; Jakobsen, et al., 2011; Jørgensen, et al., 2005c; Walcher, et al., 2014), regardless of using either a traditional or molecular quantification approach. The CPS levels in this study range from 1.5 to 3.0 log CFU/ml, a result that corresponds to levels in the above mentioned studies, and, as expected, the levels declined after pasteurization. In mature cheese, CPS were only detected sporadically in pasteurized production, and reduced to a negligible level in cheese at expiry date and at counts below bacterial levels required for enterotoxin production. The *S. aureus* was present in the whey from pasteurized production, either due to recontamination during the manual production of the cheese or to an insufficient pasteurization temperature or time period. In the current study, all farm dairies use a starter culture which ensures a lower frequency and levels of *S. aureus* (Rosengren, et al., 2010). Lactic acid bacteria (LAB) in starter cultures are also able to induce a viable but nonculturable (VBNC) form of *S. aureus* (Schellenberg, Smoragiewicz, & Karska-Wysocki, 2006) which later has a potential to regrow (Oliver, 2005), and may also give rise to the sporadic contamination of *S. aureus* that was observed in this study.

In only one production plant producing raw milk cheese was CPS detected throughout the whole cheese making process from the raw milk to the cheese at expiry date. This farm dairy produces non-pasteurized semi-hard cheese. Mean CPS levels varied between 2.2 and 3.1 log CFU/ml or /g in raw milk, whey, or cheese from this farm dairy during the cheese making process. According to other studies, maximum levels of *S. aureus* have been found 5-6 hours into the process (Duquenne, et al., 2016; Jakobsen, et al., 2011). At an initial concentration of 3.0 log CFU/ml, *S. aureus* can grow to above 5 log CFU/ml during the first 6 hours of milk

renneting (Duquenne, et al., 2016). A limited acidification from the starter culture during the processing of semi-hard cheese will support the growth of this pathogen (Duquenne, et al., 2016). The levels of *S. aureus* during the cheese making at farm B can reach levels associated with enterotoxin production, with optimal growth conditions since the generation time for *S. aureus* in milk is reported to be 0.8 hours at 25 ºC (Le Marc, Valík, & Medveďová, 2009).

A considerable part of the isolates (87.5%) in this study contained one or more SE-genes, with the largest part of the isolates from farm B, a producer of raw milk cheese. Variations in toxin gene prevalence in isolates from milk and cheese have been reported in several recent studies, from 74% down to 16% (Carfora, et al., 2015; Hummerjohann, Naskova, Baumgartner, & Graber, 2014; Hunt, Schelin, Rådström, Butler, & Jordan, 2012; Rosengren, et al., 2010). The abundancy of *sec* and *sec* combined with *tst,* as in the present study, was not surprising since *sec* has been the predominant enterotoxin gene in earlier studies. Not only of bovine bulk milk (D'Amico & Donnelly, 2010; Jørgensen, et al., 2005c), goat milk (Spanu, et al., 2012; Xing, et al., 2016), and cheese (Hunt, et al., 2012), but also of isolates from bovine mastitis (Oliveira, Rodrigues, Hulland, & Ruegg, 2011). The *sec* was present in 7 of the 16 enterotoxin profiles and present in all sample types; however, in milk environments the production of this enterotoxin can be reduced (Even, et al., 2009). In combination with *sec*, ~~a~~ considerable part of the analysed isolates often contained the *tst* gene, where the corresponding protein (TSST-1) can cause toxic shock syndrome, and is often related to isolates that cause foodborne disease (Adesiyun, Lenz, & Schaal, 1992).

The co-detection of *sec*/*tst* and *seg/sei* is consistent with the tandem location on the mobile genetic elements as *S. aureus* pathogenicity islands (*Sa*PI) and enterotoxin gene clusters (*egc*) (Alibayov, et al., 2014; Jørgensen, et al., 2005c). Moreover, *seg* and *seh* were identified in a majority of the isolates, and both have clearly been associated with food poisoning (Hennekinne, et al., 2012). One example of this was mashed potato made with raw milk

285 which contained SEH, and was served to children in a kindergarten (Jørgensen, et al., 2005a)
286 In contrast, the most common enterotoxin found in *S. aureus* isolates worldwide, and most
287 often responsible for outbreaks of SFP, is *sea* (Argudín, et al., 2010; Johler, et al., 2015;
288 Kérouanton, et al., 2007), which was only detected together with *seb* in one isolate each in
289 this study. The enterotoxin, *sed*, detected in another European study (Rola, Czubkowska,
290 Korpysa-Dzirba, & Osek, 2016) was not found in milk, whey, or cheese in this study.

291 Most of the isolates containing enterotoxin genes were from raw milk, as the amounts of CPS
292 were significantly reduced during pasteurization. The *S. aureus* isolates were only detected in
293 some whey and cheese samples, all of which came from farm B (raw milk cheese) and farm D
294 (pasteurized cheese). One explanation could be that inadequate pasteurization or
295 recontamination occurred at farm D. Insufficient acidification stimulating continuous growth
296 of the pathogen may account for the S. *aureus* contamination at farm B. At farm A, which
297 produced soft cheese from raw milk, only one isolate was found in the raw milk. One can
298 hypothesize that producers of soft, raw cheese are probably more attentive to production risk
299 factors than are other artisan cheese makers.

300 The genetic characterization revealed one dominant PFGE profile in 57% of the isolates
301 within all farm dairies and all sample types. Genetic similarity, or relatedness among *S.*
302 *aureus* isolates within a larger geographical region, was observed in studies from bulk milk in
303 sheep, goats and cows (Jørgensen, Mørk, Caugant, Kearns, & Rørvik, 2005d; Mørk,
304 Tollersrud, Kvitle, Jorgensen, & Waage, 2005; Rosengren, et al., 2010; Vautor, et al., 2003;
305 Xing, et al., 2016). Different regions may have different prevalences for *S. aureus* subtypes,
306 as indicated by Hummerjohann et al. (2014). For dairy cows and sheep, it has been shown that
307 several *S.aureus* strains are shared within and between many herds, even over long distances
308 (Vautor, et al., 2009).

Several SE gene profiles were identified within the large group of isolates with a similar PFGE profile and made it possible to further differentiate the isolates. Various PTs carried the same SE gene profile, suggesting that an association between the two characteristics may not be present. In contrast, earlier studies have observed that *S. aureus* isolates with indistinguishable PFGE profiles had the same SE gene profile and could not be further differentiated (Loncarevic, Jørgensen, Løvseth, Mathisen, & Rørvik, 2005). Earlier studies have also grouped together identical SE genotypes (Jørgensen, et al., 2005d). A possible lack of association between PT and SE gene profiles has been suggested (Oliveira, et al., 2011; Yanping, et al., 2011).

The large group of isolates with similar PFGE profiles could be even further differentiated with *spa*-typing, concordant with the discrimination of MRSA isolates with identical PFGE profiles in a recent study (Church, Chow, Lloyd, & Gregson, 2011). However, *spa*-typing alone was not able to distinguish all of the isolates in the present study to as high a degree as those with PFGE. Because of its discriminatory power, PFGE has been regarded as the standard method of characterizing diversity in *S. aureus,* especially in foodborne outbreaks (Kérouanton, et al., 2007). The *spa*-typing is easier to perform, but limited by the fact that it is a single–locus typing approach, and is thereby less discriminatory than multi-locus sequencing methods (Aires-de-Sousa, et al., 2006). Seven different *spa* types were detected in the current study, indicating that *spa*-typing may not be a useful method for bovine isolates, as suggested by Ikawaty et al. (2009). The predominant *spa* type in this material, t2678, belongs to MLST CC133 and ST133 in sheep and goats (Porrero, et al., 2012), since *spa* types are normally associated with specific MLST types (Hasman, et al., 2010; Strommenger, et al., 2006). This is also the most common *S. aureus* clone within ruminants in Norway (Jørgensen, et al., 2005d; Mørk, Kvitle, & Jørgensen, 2012) and the second most common in Spain (Porrero, et al., 2012). Of the other identified *spa* types in this study, t127 has been associated

334 earlier with human isolates (Hummerjohann, et al., 2014). Recently, t127 was identified in
335 sheep milk, possibly originating from humans milking the sheep with bare hands (Carfora, et
336 al., 2016), and earlier this *spa* type was identified in pigs (Normanno, et al., 2015). The *spa*-
337 type t127 carrying *seh* has been linked to SFP in a recent study (Roussel, et al., 2015), which
338 additionally demonstrates the genetically related background among isolates carrying *seh.*

339 This study clearly shows that one typing method alone is not sufficient to fully discriminate *S.*
340 *aureus* isolates from milk and cheese, and that, e.g., *spa*-typing could be used in combination
341 with additional markers as suggested by earlier studies (Strommenger, et al., 2008).

342 Multiple sources of contamination were reflected in the high genetic diversity among isolates
343 from the same raw milk samples, and especially from the cheeses from farms B and D,
344 confirming the contamination from more than one source, as shown in other studies (Carfora,
345 et al., 2015; Hummerjohann, et al., 2014; Loncarevic, et al., 2005; Rosengren, et al., 2010).
346 Farm cheese is manually manufactured, and can be contaminated by both workers and the
347 environment unless strict production practices are followed. The variation of isolates on farms
348 is usually regarded as low in studies from cows (D'Amico & Donnelly, 2011; Mørk, et al.,
349 2005), even if contamination can spread between the farm dairies, as indicated by Xing and
350 coworkers (2016). According to the questionnaire, however, there is no contact between the
351 different dairies except for farms B and D, which sporadically exchanged spices to be added
352 during cheese production.

353 In the present work, none of the isolates contained the *mecA* gene, and all isolates were
354 susceptible to all the antibiotics tested, except for the low prevalence of the *blaZ* gene. This
355 reflects both the restrictive attitude to the use of antibiotics and the effective vaccination of
356 farm animals in Norway (NSCFS, 2015). Due to the low prevalence of MRSA in humans, the
357 authorities perform an active search for MRSA contaminated livestock, followed by

destruction of the herd if detected. (Sunde, et al., 2011).The amount of antimicrobial agents used in Norwegian agriculture is very low compared to that of other countries (ESVAC, 2015). Nevertheless, MRSA has been detected in pigs in different regions (NSCFS, 2015) and maintaining the situation is a constant struggle. The potential for its spread to farm dairies through either humans or the exchange of livestock animals is possible (NFSA, 2015), especially in areas with a high livestock density of both cows and pigs (NFSA, 2015; Locatelli, et al., 2016)

This study points out that the production of raw milk cheeses encounters an increasingly challenging situation. In particular, that artisan cheese making might boost the possible risk of SFP-illness. Artisan cheese makers, despite having a general awareness of microbial hazards, have limited ability to assess and manage risks (Le, Bazger, Hill, & Wilcock, 2014). Even pasteurized production is at risk since most artisan producers pasteurize the milk in the vat, where temperature control is notoriously difficult.

5 Conclusion

Genetically related *S. aureus* are present in all of the raw milk samples from the farm dairies producing different cheese types. They can produce different enterotoxins that may cause SFP. Farm dairies are located in close proximity to both the farm and wild animals. Sampling the production stages from raw milk to cheese contributes to our understanding of how *S. aureus* contamination pathways occur, and thus enable targeted control measures throughout the cheese making process. In the present study, a large group of *S. aureus* isolates with similar PFGE profiles dominated in the samples of milk, whey, and cheese from five artisan farm dairies. This study clearly shows that one typing method alone is not sufficient to fully discriminate *S. aureus* isolates from milk and cheese. The prevalence of *S. aureus* in raw milk and of enterotoxin genes is high, and this study revealed a high diversity in the isolates

carrying enterotoxin genes. This reveals the lurking threat of SFP that can surface when conditions are favourable.

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Table 1 Summary of production information from the questionnaire from the participating farm dairies

Farm dairy	**A**	**B**	**C**	**D**	**E**
Cheese type	Soft	Semi-hard	Semi-hard	Soft	Soft
Pasteurized product	No	No	Yes	Yes	Yes
Cheese production (kg/year)	500	1700	4000	12 000	5000
Supplementary milk from other farms	No	No	No	Yes	No
Exchanges ingredients with other producers	No	Yes	No	No	No
Water service provision	Public	Private	Private	Public	Public
*Frequency of mastitis problem**	Seldom	Occasionally	Occasionally	Seldom	Seldom

* seldom ≤ 1 p.a., occasionally ≥ 2 p.a.

Table 2 A. Coagulase positive staphylococci (CPS) in milk, whey and cheese samples from five different farm dairies (n=3, in each group). **B.** Summary of CPS during the cheesemaking process in this study. Estimates of CPS per ml milk or per g cheese are preformed using log-transformed data, and counts equal to zero were scored as 1 CFU/g or ml.

A

Milk treatment	**Cheese type**	**Farm dairy**	**Raw milk** log CFU/ml	**Cheese Milk** log CFU/ml	**Whey** log CFU/ml	**Cheese** log CFU/g	
						mature	at expiry date
Non-pasterized	Soft	A	2.0 ± 0.6		0.6 ± 1.1	0.9 ± 1.5	0.0 ± 0.0
	Semi-hard	B	2.3 ± 0.2		2.2 ± 0.6	3.1 ± 0.70	2.2 ± 1.9
Pasteurized	Semi-hard	C	2.6 ± 0.11	0.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Soft	D	2.7 ± 0.59	0.3 ± 0.6	0.5 ± 0.8	1.2 ± 2.2	0.0 ± 0.0
	Soft	E	1.7 ± 0.23	0.0 ± 0.0	0.6 ± 0.98	0.9 ± 1.6	0.0 ± 0.0

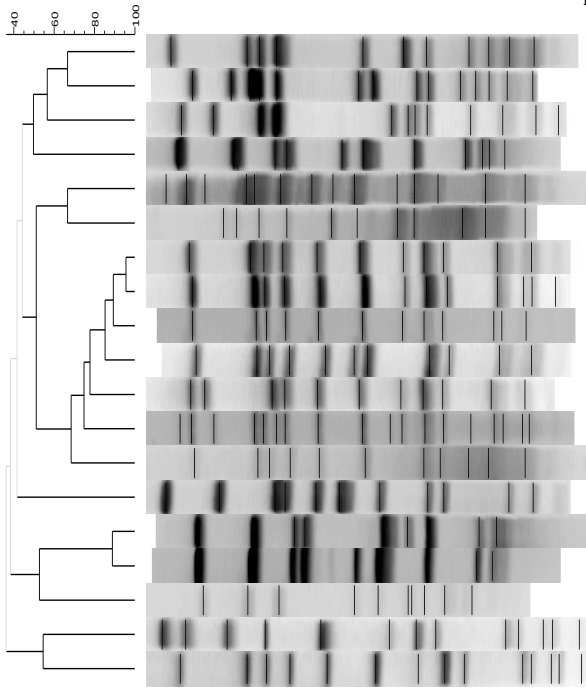
B

Samples	**Positive samples in %**	**Mean (range) level** (log CFU/ml or /g)[1]
Raw milk (n=15)	100	2.3[a] (1.5-3.0)
Pasteurized milk for cheesemaking (n=9)	22	0.2[c] (0.0-1.0)
Whey, non-pasteurized production (n=6)	67	1.4 [a, b, c] (0.0-2.5)
Whey, pasteurized production (n=9)	22	0.3[c] (0.0-1.7)
Cheese, mature, non-pasteurized (n=6)	67	2.0 [a,b] (0.0-3.5)
Cheese, mature, pasteurized (n=9)	22	0.7 [b,c] (0.0-3.5)
Cheese, at expiry date,non-pasteurized (n=6)	33	1.1 [a,b,c] (0.0-3.5)
Cheese, at expiry date, pasteurized (n=9)	0	0.0 [c] (0.0-0.0)

1: CPS levels with different superscript letters (a, b, c) have means that are significantly different ($p<0.05$).

Table 3 Enterotoxin gene profiles in *S. aureus* isolates (n=40) from five farm dairies (A to E). Isolates are from raw milk, pasteurized (p) or nonpasteurized (np), whey, mature cheese (cheese ma) or cheese at expiry date (cheese ex).

Toxin gene profile		Sample type	A	B	C	D	E	Total
I	None	raw milk (p), whey		2	1	1	1	5
II	*sec*	raw milk (p+np)	1	1		1		3
III	*seg*	raw milk, cheese ex				1	1	2
IV	*seh*	raw milk, whey		1	1	1	1	4
V	*sec, tst*	raw milk (p+np), whey		4	1	5		10
VI	*seg, tst*	raw milk (np)		1				1
VII	*seg, seh*	raw milk, cheese ex		1			1	2
VIII	*seg, sei*	raw milk					1	1
IX	*seh, tst*	whey		1				1
X	*sea, sec, tst*	raw milk (np)		1				1
XI	*seb, sec, tst*	whey				1		1
XII	*sec, seg, tst*	whey, cheese ma		2				2
XIII	*sec, seh, tst*	cheese ma				1		1
XIV	*seg, seh, sei*	raw milk (p)			1		1	2
XV	*seg, seh, tst*	raw milk (p)				1		1
XVI	*sec, seg, seh, tst*	raw milk (p+np)		2	1			3
Total			1	16	5	12	6	40



Representative isolates ID	PT	Spa	Farm	No of isolates	Sample type	Toxin gene profile
227	01	t197	E	3	raw milk	I,VII
133	02	t127	E	2	raw milk	IV
160	03	t346	D	1	raw milk	IV
192	04	NA	C	1	raw milk	IV
258	05	t2678	B	1	whey	XII
256	06	t2678	B	1	raw milk np	XVI
182	07.1	t2678	A, B,C,D	9	raw milk, whey	I, II, V, XI
169	07.2	t2678	B, C	4	raw milk, whey	V
177	07.3	t346, t544, t2678	B, C, D	27	raw milk, whey, cheese ma	II, III, IV, V, IX ,X ,XIII, XIV
217	07.4	t3495	E	4	raw milk	III
151	07.5	t2678	D	2	raw milk	II
161	07.6	t346	B	1	whey	I
294	08	t2678	B	1	cheese ma	XII
232	09	t2873	C, E	5	raw milk	XIV, VIII
126	10,1	NA	D	1	raw milk	I
127	10,2	t346	D	2	raw milk	V
301	11	t2678	B	1	cheese ex	VII
254	12	t2678	B	2	raw milk, whey	VI, I
115	13	t2678	D	1	raw milk	XV

Figure 1 PFGE profiles and dendrogram of representative PFGE pulsotypes (PT) of *S. aureus* isolated from milk, whey and cheese samples from five dairy farms (A to E). *Spa* typing and toxingene profile screening was performed on selected isolates. Isolates are from raw milk, non-pasteurized (np), whey, mature cheese (cheese m) or cheese at expiry date (cheese ex).

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