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# Molecular characterisation and biofilm production in *Staphylococcusaureus* isolates from the dairy production chain in Northern Italy

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ABSTRACT

*Staphylococcus aureus* is able to produce enterotoxins causing staphylococcal food poisoning, and is frequently harboured by dairy products. Also, *S. aureus* is able to form biofilm in the production environment, enhancing the risk of food contamination. The ability of 49 *S. aureus* isolates from the dairy production chain to form biofilm aim was tested, and their genetic diversity in terms of population structure and presence of genes involved in biofilm formation or enterotoxins production was explored. The majority of the genotypes found were generally bovine associated; however, some have been also reported frequently in human clinical cases. Two isolates were methicillin-resistant. In total, 38.7% of the isolates were biofilm producers, and among them 47.3%, 42.1% and 10.5% exhibited weak, moderate, or strong biofilm-forming ability, respectively. In total 68% of the biofilm producing isolates were also positive for enterotoxins genes, raising concerns for consumer safety.



## 1. Introduction

*Staphylococcus aureus* (*S. aureus*) is a facultative anaerobic Gram-positive coccus that can be isolated from a wide range of food, animal, human, and medical environments (Lee, Bae, Lee, & Lee, 2015). *S. aureus* can produce staphylococcal enterotoxins (SEs) causing the most prevalent foodborne intoxication worldwide, namely, staphylococcal food poisoning (SFP) (Johler et al., 2015). In farms, *S. aureus* is the major mastitis pathogen, able to spread among cows through contact with contaminated equipment, and causing significant losses in the dairy industry (Fessler et al., 2010; Silva et al., 2013; Voelk et al., 2014).

Pulsed-field gel electrophoresis (PFGE) has long been regarded as the gold standard for *S. aureus* typing; however, the lack of an internationally shared nomenclature limits the use of this technique for epidemiology and population description (Chua, Howden, Jiang, Stinear, & Peleg, 2014). Multi-locus sequence typing (MLST), which involves sequencing of seven "housekeeping" genes present in all strains of *S. aureus*, and comparison with an online database to identify a sequence type (ST), is at present the most widely accepted typing method (Chua et al., 2014). A less laborious and costly sequence-based method is *spa* typing, based on the sequence variability in the polymorphic repeat region of the *S. aureus* protein A gene (*spa*) (Shopsin et al., 1999). Generally, *spa* typing is more discriminatory than MLST and the *spa* types broadly correspond to MLST based clonal complexes (Cookson et al., 2007). MLST and *spa* typing allow easy comparison of strains between laboratories through online databases, which can also be used to submit isolates sequences to designate new types (Enright, Day, Davies, Peacock, & Spratt, 2000; Harmsen et al., 2003). RS-PCR is another typing technique based on the PCR amplification of the ribosomal spacer (RS) (Graber et al., 2009). This technique has proved useful in discriminating *S. aureus* strains with different virulence potential (Cosandey et al., 2016).

Methicillin-resistant *S. aureus* (MRSA) harbour antibiotic-resistance genes, and are recognised through the detection of *mecA*, or its homologue *mecC*, a gene responsible for resistance to

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methicillin (García-Álvarez et al., 2011). MRSA were historically related to the nosocomial environment; however, in recent years they have been isolated from various foods of animal origin, including dairy products, and in bovine mastitis cases, raising alarms about possible dissemination throughout the food production chain (Kamal, Bayoumi, & Abd El Aal, 2013; Normanno et al., 2007). MRSA can cause severe infections to humans, and therefore, have been more thoroughly investigated, compared with methicillin-susceptible *S. aureus* (MSSA) (Chua et al., 2014). However, MSSA can still pose a risk, due to the production of SEs. Dairy products can be a vehicle of transmission of enterotoxigenic *S. aureus* strains, and are frequently involved in SFP outbreaks (EFSA, 2015; Hennekinne, De Buyser, & Dragacci, 2012).

Many SFPs are caused by food-handlers acting as carriers during food processing (Rola, Czubkowska, Korpysa-Dzirba, & Osek, 2016), but also the presence of mastitis in the herd or characteristics of the milking environment can be sources of contamination relevant to the dairy production chain (Kümmel et al., 2016). Indeed, *S. aureus* is a highly adaptable bacterium, which has the ability to form biofilms, microbial communities formed by cells that are strongly attached to a surface while embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002). In this respect, *S. aureus* biofilms can be frequently found in food processing plants, and biofilm formation can protect the microorganism from sanitation procedures and environmental stresses (Abdallah, Benoliel, Drider, Dhulster, & Chihib, 2014).

Specifically related to the dairy industry, raw milk has a natural microbiota that can perform initial attachment to surfaces favouring the anchoring of contaminant bacteria to the biofilm, and raising concerns for food safety (Marchand et al., 2012). Many studies have investigated the ability of MRSA from the dairy sector to form biofilm. However, most studies are focused on isolates collected from the herd, and not from the final products, which are more relevant in a food safety perspective. Moreover, since also MSSA can pose a risk for the consumer due to the production of SEs, it is important to investigate their ability to form biofilm and become contaminants (Bardiau et al., 2013; Prenafeta, Sitja, Holmes, & Paterson, 2014). Thus, the present study was carried out to (i) explore the genetic diversity of *S. aureus* isolated from dairy products in Northern Italy through MLST, *spa* typing and RS-PCR to provide comparable results and hypothesis on the source of contamination, (ii) detect the presence of SEs genes, (iii) detect relevant genes involved in biofilm formation, and (iv) determine the ability of isolates to produce biofilm. Finally, we attempted to evaluate the potential relationship among specific molecular types and biofilm phenotype of dairy isolates. Overall, no specific correlation between genotype and biofilm production was found. However, clones both from the animal and human reservoir have been found throughout the production chain, indicating multiple sources of contamination.

## 2. Materials and methods

### 2.1. Samples selection and S. aureus isolation and identification

*S. aureus* isolates were collected from products tested during routine surveillance carried out by the Regional Laboratory for Animal Heath and Food Safety (IZSLER), or through ad hoc sampling carried out by the Department of Food and Drug of the University of Parma. The samples were collected between 2014 and 2016 in 33 plants located in Northern Italy (Lombardy and Emilia Romagna Regions), of which 28 were small scale artisanal dairies located in pastures, and five were industrial dairies. For *S. aureus* isolation, serial dilution of each sample homogenate were plated on Baird Parker agar + rabbit plasma fibrinogen (BP-RPF) (Biolife Italiana, Milano, Italy) and incubated at 37 °C for 48 h. Up to 5 characteristic colonies for each sample were plated on blood agar to confirm *S. aureus* haemolytic property. A total of 49 representative isolates (n = 13 from raw milk, n = 5 from curd, n = 25 from cheese, and n = 6 from food contact surfaces), were selected for further characterisation (Table 1). The species identification was confirmed with PCR of the *nuc* gene as described by Brakstad, Aasbakk, & Maeland, 1992. DNA was obtained by boiling a suspension of one isolated colony from blood agar in 100 μL 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 following PCR assays. The *S. aureus* isolates and their boiled extracts were then stored at −80 °C.

### 2.2. Molecular characterisation

#### 2.2.1. Multilocus sequence yyping and spa typing

All isolates were typed with MLST as described by Enright et al. (2000); the sequence types (STs) were determined with the database available on the *S. aureus* MLST website (https://pubmlst.org/saureus/) sited at the University of Oxford (Jolley & Maiden, 2010). For *spa* typing, the *spa* gene was amplified by PCR as described by Shopsin et al. (1999), and *spa* types were determined with the

**Table 1**
List of the *S. aureus* isolates analysed indicating the source and plant of production.[a]

| ID | Source | Plant | ID | Source | Plant | ID | Source | Plant |
|---|---|---|---|---|---|---|---|---|
| A | Raw milk | 1 | B3 | Cheese | a1 | B19 | Fresh cheese | a15 |
| B | Vat | 1 | B4 | Cheese | a1 | B20 | Fresh cheese | a16 |
| C | Vat | 1 | B5 | Curd | 4 | B21 | Raw milk | a17 |
| D | Drying plank | 1 | B6 | Cheese | a2 | B22 | Fresh cheese | a18 |
| E | Drying plank | 1 | B7 | Cheese | a3 | B23 | Fresh cheese | a19 |
| F | Drying plank | 1 | B8 | Raw milk | a4 | B24 | Fresh cheese | a20 |
| G | Drying plank | 1 | B9 | Curd | a5 | B25 | Raw milk | a21 |
| H | Cheese | 1 | B10 | Fresh cheese | a6 | B26 | Fresh cheese | a22 |
| I | Cheese | 1 | B11 | Curd | a7 | B27 | Raw milk | a23 |
| J | Cheese | 1 | B12 | Fresh cheese | a8 | B28 | Raw milk | a24 |
| K | Cheese | 1 | B13 | Fresh cheese | a9 | B29 | Fresh cheese | a25 |
| L | Cheese | 1 | B14 | Raw milk | a10 | B30 | Curd | a26 |
| M | Cheese | 1 | B15 | Fresh cheese | a11 | B31 | Curd | a27 |
| N | Cheese | 1 | B16 | Raw milk | a12 | B32 | Raw milk | 5 |
| O | Cheese | 1 | B17 | Raw milk | a13 | B33 | Fresh cheese | a10 |
| B1 | Raw milk | 2 | B18 | Fresh cheese | a14 | B34 | Raw milk | a28 |
| B2 | Raw milk | 3 | | | | | | |

[a] Plants preceded by the "a" letter correspond to alpine artisanal dairies.

Ridom StaphType software (Ridom GmbH, Würzburg, Germany). All DNA sequences were obtained with a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Novel MLST and *spa* sequences were submitted to the respective database for the designation of the new profile. MLST data were analysed with the goeBURST algorithm using the Phyloviz software (Francisco et al., 2012).

### 2.2.2. Detection of mecA and mecC

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. (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 boiled extract. 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.

### 2.2.3. Detection of staphylococcal enterotoxins

Two multiplex PCR protocols were used as described in Bianchi et al. (2014) to detect *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sep*, and *ser* SEs genes.

### 2.2.4. Detection of genes involved in biofilm production

Genotyping of the *S. aureus* accessory gene regulator (*agr*) was conducted by multiplex PCR amplification of the hypervariable domain of the *agr* locus using a single forward primer and 4 reverse primers specific for each of the 4 major specificity groups (*agr* I to IV), according to Shopsin et al. (2003). PCR assays were conducted on genes encoding for intercellular adhesion (*icaA* and *icaD*), the collagen binding protein (*cna*), the clumping factor A (*clfA*), the fibronectin binding proteins A and B (*fnbA* and *fnbB*), the α and β hemolysins (*hla* and *hlb*), and the regulator protein (*sarA*). Primers used in this work were synthesised by Integrated DNA Technologies (IDT, Coralville, Iowa, USA; Table 2). Gene amplification was performed as described by Graber et al. (2009), with some modifications. Briefly, the PCR reaction mix (total volume of 25 μL) for amplification of the *icaA*, *icaD*, *cna*, *fnbA*, *fnbB*, *clfA*, *hla*, *hlb*, and *sarA* genes contained 1 × Maxima Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1 μM of each primer, and 5 μL of boiled extract. For the *agr* genotyping, multiplex PCR assays were conducted using 0.3 μM of each primer. The PCR profile for *agr* genotyping was 95 °C for 5 min, followed by 35 cycles comprising 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. Then *icaA*, *icaD*, *cna*, *fnbA*, *hla*, and *sarA* were amplified using the following PCR cycle: 95 °C for 5 min, followed by 40 cycles comprising a denaturation step at 95 °C for 40 s, followed by the annealing step at 50 °C for 40 s and extension at 72 °C for 40 s. The final elongation step was performed at 72 °C for 10 min. The PCR conditions for *clfA*, *fnbB*, and *hlb* amplification were 95 °C for 5 min followed by 35 cycles of 95 °C for 35 s, 50 °C for 35 s, and 72 °C for 1 min. The final elongation step was performed at 72 °C for 10 min. Negative and positive controls were included in every run for all the different PCRs. Nucleic acid of *S. epidermidis* ATCC 12228 DNA was used for the negative control, and *S. aureus* ATTC 35556 DNA (Gene Bank; NCBI Reference Sequence: NC_007795.1) as the positive control. All PCR reactions for the detection of virulence genes were performed using a Techne TC- 412 thermal cycler (Bibby Scientific Limited, Staffordshire, UK). PCR assays were visualised by agarose electrophoresis (1% agarose gel in Tris-acetate-EDTA buffer) and GelRed (Biotium, Hayward, CA, USA) staining.

### 2.2.5. Biofilm formation assay

For the evaluation of biofilm production three strains from a culture collection (ATCC3556, ATCC12600, ATCC12228; American Type Culture Collection) were used as reference. All dairy isolates (n = 49) and the reference strains were tested in triplicate on 6 wells Nunclon™ Delta Surface polystyrene tissue culture plates (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for biofilm production. Biofilm formation was evaluated following a previously described method (Di Ciccio et al., 2015). Before each experiment, *S. aureus* isolates from the frozen stocks (−80 °C) were grown overnight at 37 °C in 5 mL tryptic soy broth (TSB; Oxoid, S.p.A., Milan, Italy) and then plated on tryptic soy agar (TSA; Oxoid). Briefly, fresh cultures from TSA plates were suspended in TSB and incubated at 37 °C overnight. Cultures were then washed three times with PBS (pH 7.3, Sigma–Aldrich S.r.l., Milan, Italy) and diluted to desired inoculum concentration ($10^8$ cfu $mL^{-1}$) with fresh TSB based on the optical density (OD) at 550 nm (Varian SII Scan Cary 100 spectrophotometer - Agilent Technologies, Santa Clara, CA). Three millilitres of the standardised inoculum was then added to polystyrene tissue culture plates (961 $mm^2$, ⌀ 35 mm). Samples were then incubated at 37 °C for 24 h. After incubation, non-adherent cells were removed by dipping each sample 3 times in sterile PBS. Samples were fixed at 60 °C for 1 h and stained with 3 mL of 2% crystal violet solution in 95% ethanol for 15 min. After staining, samples were washed with distilled water. Negative controls underwent the same treatment, without inoculation.

The quantitative analysis of biofilm production was performed by adding 3 mL of 33% acetic acid to de-stain the samples. From each sample, 200 μL was transferred to a microtiter plate and the OD level of the crystal violet solution present in the de-staining solution was measured at 492 nm (Victor, Perkin Elmer, Waltham,

**Table 2**
Oigonucleotide primers for the biofilm related genes analysed in this study.

| Target gene | Forward primer (5′-3′) | Reverse primer (5′-3′) | Amplicon size (bp) | References |
|---|---|---|---|---|
| *agrI* | ATGCACATGGTGCACATGC | GTCACAAGTACTATAAGCTGCGAT | 441 | (Shopsin et al., 2003) |
| *agrII* | ATGCACATGGTGCACATGC | TATTACTAATTGAAAAGTGGCCATAGC | 575 | (Shopsin et al., 2003) |
| *agrIII* | ATGCACATGGTGCACATGC | GTAATGTAATAGCTTGTATAATAATACCCAG | 323 | (Shopsin et al., 2003) |
| *agrIV* | ATGCACATGGTGCACATGC | CGATAATGCCGTAATACCCG | 659 | (Shopsin et al., 2003) |
| *icaA* | ACACTTGCTGGCGCAGTCAA | TCTGGAACCAACATCCAACA | 188 | (Kouidhi, Zmantar, Hentati, & Bakhrouf, 2010) |
| *icaD* | ATGGTCAAGCCCAGACAGAG | AGTATTTTCAATGTTTAAAGCAA | 198 | (Kouidhi et al., 2010) |
| *cna* | AAAGCGTTGCCTAGTGGAGA | AGTGCCTTCCCAAACCTTTT | 192 | (Montanaro, Renata Arciola, Baldassarri, & Borsetti, 1999) |
| *fnbA* | GATACAAACCCAGGTGGTGG | TGTGCTTGACCATGCTCTTC | 191 | (Arciola, Campoccia, Gamberini, Baldassarri, & Montanaro, 2005) |
| *fnbB* | GGAGAAGGAATTAAGGCG | GCCGTCGCCTTGAGCGT | 811 | (Booth, Pence, Mahasreshti, Callegan, & Gilmore, 2001) |
| *clfA* | CCGGATCCGTAGCTGCAGATGCACC | GCTCTAGATCACTCATCAGGTTGTTCAGG | 1000 | (McDevitt, Francois, Vaudaux, & Foster, 1995) |
| *hla* | CTGGCCTTCAGCCTTTAAGG | CTGTAGCGAAGTCTGGTGAAA | 455 | (Ando, Monden, Mitsuhata, Kariyama, & Kumon, 2004) |
| *hlb* | GCCAAAGCCGAATCTAAG | CGCATATACATCCCATGGC | 845 | (Ando et al., 2004) |
| *sarA* | TTAGCTTTGAAGAATTCGCTGT | TTCAATTTCGTTGTTTGCTTC | 275 | (Padmapriya, Ramesh, Chandrashekar, & Varadaraj, 2003) |

MA). All results were expressed by calculating the biofilm production index (BPI) as follows: BPI = [$OD_{meanbiofilm}$/surface ($mm^2$)] × 1000.

Finally, all isolates were assigned to different categories based on their BPI values. For this purpose, the BPI of the two *S. aureus* and the *S. epidermidis* reference strains were used as control. Briefly, BPI was compared with reference strains: *S. aureus* ATCC35556 (BPIPC, strong biofilm producer; Seidl et al., 2008) as positive control; *S. aureus* ATCC12600 (BPI12600, moderate biofilm producer; Di Ciccio et al., 2015); *S. epidermidis* 12228 (BPINC, negative biofilm producer; Lee et al., 2015) as negative control (Table 3). The cut-off point for biofilm production was the BPI value obtained by BPINC on polystyrene (0.294). *S. aureus* strains showing the ability to produce biofilms were classified as weak (BPINC ≤ *S. aureus* BPI < BPI12600), moderate (BPI12600 ≤ *S. aureus* BPI < BPIPC), or strong (*S. aureus* BPI ≥ BPIPC).

### 2.3. Statistical analysis

For each *S. aureus* isolate the relative frequencies (%) of genes involved in biofilm production, in antibiotic-resistance and genes related to SEs were calculated, and the Clopper-Pearson method was used to compute 95% confidence interval. Correlation between genes involved in biofilm production, in antibiotic-resistance and genes related to SEs was calculate by Phi index. The association between *S. aureus* isolates and genes involved in biofilm production, in antibiotic-resistance and genes related to SEs was evaluated by a chi-square test ($\chi^2$) or Fisher exact test where appropriate. Statistically significant associations were assessed by logistic regression model and estimated OR and 95% Wald's confidence interval (CI) were obtained as measures of predictor effect. The likelihood ratio test was used to assess the overall significance of the model (two-tailed significance level $p \leq 0.05$). All analyses were performed using R software (R Development Core Team).

## 3. Results and discussion

*S. aureus* is a pathogen that causes several serious diseases in both humans and animals worldwide. In dairy processing plants, *S. aureus* contamination could be due to various sources: raw milk, processing environment, and handlers (André et al., 2008; Arcuri et al., 2010). *S. aureus* is a major concern for the food processing industry due to its virulence factors and ability to form biofilm (Langsrud, 2009; Xing et al., 2016). Previous studies have investigated the biofilm formation of *S. aureus* isolated from clinical origin or food sector (de Souza et al., 2014; Rode, Langsrud, Holck, & Møretrø, 2007). However, data on the genetic diversity and on the biofilm forming capacity of *S. aureus* isolates collected throughout the dairy production chain are limited. In this study we examined the molecular diversity and the presence an array of virulence genes (i.e., enterotoxins, adhesins, and gene regulators) of 49 isolates. We also investigated whether the biofilm-forming capacity is affected by the presence of these genes or it is correlated with different *S. aureus* types.

**Table 3**
Biofilm formation, expressed as $BPI_s$, by *S. aureus* and *S. epidermidis* reference strains on polystyrene (961 $mm^2$) at 37 °C.[a]

| Reference strains | $OD_{mean\ biofilm}$ polystyrene | BPI polystyrene |
|---|---|---|
| *S. aureus* ATCC 35556 | 0.728 ± 0.15 | 0.758 |
| *S. aureus* ATCC 12600 | 0.389 ± 0.07 | 0.405 |
| *S. epidermidis* ATCC 12228 | 0.283 ± 0.05 | 0.294 |

[a] *S. aureus* ATCC 35556 and *S. epidermidis* ATCC 12228 were positive and negative controls, respectively. Values for $OD_{mean\ biofilm}$ polystyrene are expressed as OD mean ± standard deviation.

### 3.1. Molecular typing

The MLST identified 14 different Sequence Types (STs) among the isolates tested (Table 4). We identified two novel STs, namely ST4104 and ST4162. The most commonly observed ST was ST8 (n = 17, 35%), followed by ST71 and ST97 (n = 8, 16%, and n = 6, 12%, respectively). The BURST algorithm assigned the STs into ten groups, including one Clonal Complex (CC) with ST97 as founder (composed by 18 isolates; 37%), and 9 singletons (Fig. 1). The *spa* typing identified 22 *spa* types, including 3 novel *spa* types, namely t16951, t16952 and t17143. The most commonly observed *spa* types were t2953, corresponding to ST8, and t254, corresponding to ST71 (9 isolates each, 18%; Fig. 1).

The majority of the STs found, are previously reported to be bovine associated (https://pubmlst.org/saureus/; Feltrin et al., 2015). However, even if generally specific lineages are adapted to particular mammalian hosts, some lineages have a broad host range (i.e., ST1) and host shifts have also been reported (i.e., CC97, ST8) (Budd et al., 2015; Feltrin et al., 2015; Lozano et al., 2011; Resch et al., 2013). As an example, CC97 is currently regarded as one of the major *S. aureus* CCs in bovines, and it has been reported among pig and dairy cattle holdings also in Italy (Feltrin et al., 2015). Nevertheless, CC97 has been lately reported as the cause of human pandemics, demonstrating the ability to spread to the human host (Lozano et al., 2011; Spoor et al., 2013). Within CC97, it has been reported that ST71 evolved into a distinct subgroup, which may more efficiently infect the bovine udder due to the resistance acquired against the antibiotics most commonly used for mastitis treatment (Budd et al., 2015). The CC97 subgroup represented by ST71, and its double-locus variant (DLV) ST3078, correspond to genotype R (GTR) and its variants as defined by RS-PCR (Table 4). In a previous study, these genotypes were observed in several European countries with the exception of Italy (Cosandey et al., 2016).

The other major cluster of isolates was represented by ST8. Among ST8 isolates, 15 out of 17 (88%) were GTB or GTB variants (Table 4). GTB, which has been reported to be more virulent than the other GTs, is the most widespread bovine associated *S. aureus* type in Switzerland and central Europe countries (Cosandey et al., 2016). It is believed MSSA CC8 strains have adapted themselves to the bovine host following a human-to-cow shift, and the possibility for these strains of acquiring methicillin resistance is of concern for both the human and animal health (Resch et al., 2013).

Among the 49 isolates analysed, two were MRSA (*mecA*+; Table 4), and both belonged to the ST1 cluster (2/3, 67%). CC1 MRSA are present worldwide and adapted to both the animal and the human host (Feltrin et al., 2015; Giuffrè et al., 2012).

### 3.2. Detection of enterotoxins

At least one SEs gene was found in 63% of the isolates (n = 31) and 14 different SEs genes profiles, which were overall homogenous within the different STs, were distinguished (Tables 4 and 5). None of the isolates was positive for *seb* and *sec* genes, while *sed* gene, found in 22 (47%) of the isolates, was the most frequent, followed by *ser* (n = 14; 29%) and *sej* (n = 12; 24%) genes. In particular, almost all ST8-GTB isolates contained the SE gene pattern *sed*, *sej*, *ser*, which are carried on the same plasmid (Bianchi et al., 2014), with about half of them (9/17) additionally carrying *sea*, as also reported in other studies (Hummerjohann, Naskova, Baumgartner, & Graber, 2014). Notably, it has been reported that CC97 (or GTR and its variants) isolates were characterised by the lack of SEs genes (Budd et al., 2015; Cosandey et al., 2016), while in

**Table 4**
Results of the molecular typing and biofilm production assay for the 49 S. *aureus* isolates analysed in this study.[a]

| ID | ST | CC | *spa* type | GT | BPI | Regulators | | Adhesins | | | | | Haem *hla* | MRSA *mecA* | Enterotoxins | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *agr* | *sarA* | *icaA* | *icaD* | *cna* | *fnbB* | *clfA* | | | *sea* | *sed* | *see* | *ser* | *seg* | *seh* | *sei* | *sej* | *sep* |
| B2 | 1 | 1 | t127 | BJ | + | III | + | + | + | + | − | + | + | − | − | − | − | − | − | + | + | − | − |
| B4 | 1 | 1 | t127 | BJ | ++ | III | + | + | + | + | − | + | + | + | − | − | − | − | − | + | − | − | − |
| B26 | 1 | 1 | t127 | ND | + | III | + | + | + | + | + | + | + | + | − | − | − | − | − | + | − | − | − |
| E | 8 | 8 | t2953 | B | − | I | + | + | + | + | + | + | − | − | − | + | − | + | − | − | − | + | − |
| F | 8 | 8 | t2953 | B | − | I | + | + | + | + | + | + | + | − | + | − | − | + | − | − | − | + | − |
| H | 8 | 8 | t2953 | B | − | I | + | + | + | + | + | + | − | − | − | + | − | + | − | − | − | − | − |
| B6 | 8 | 8 | t2953 | B | ++ | I | + | + | + | + | + | + | + | − | + | + | − | + | − | − | − | + | − |
| B7 | 8 | 8 | t2935 | B | + | I | + | + | + | + | + | + | + | − | + | + | − | + | − | − | − | + | − |
| B14 | 8 | 8 | t2953 | B | ++ | I | + | + | + | + | + | + | + | − | + | + | − | + | − | − | − | + | − |
| B16 | 8 | 8 | t2953 | B | − | I | + | + | + | − | + | + | + | − | + | + | − | + | − | − | − | + | − |
| B25 | 8 | 8 | t2935 | B | + | II | + | + | + | + | + | + | + | − | − | − | − | + | − | − | − | − | − |
| B33 | 8 | 8 | t2953 | B | − | I | + | + | + | + | + | + | − | − | + | + | − | + | − | − | − | + | − |
| B34 | 8 | 8 | t2953 | B | − | I | + | + | + | + | + | + | − | − | + | + | − | + | − | − | − | + | − |
| B17 | 8 | 8 | t13265 | B | + | I | + | + | + | + | + | + | + | − | − | + | − | + | − | − | − | + | − |
| B8 | 8 | 8 | t024 | B | + | I | + | + | + | + | + | + | + | − | + | − | − | − | − | − | − | − | − |
| B | 8 | 8 | t164 | B | − | I | + | + | + | + | + | + | − | − | − | + | − | + | − | − | − | + | − |
| O | 8 | 8 | t3802 | B | − | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B18 | 8 | 8 | t3802 | AA | + | I | + | + | + | + | + | + | + | − | + | + | − | + | − | − | − | + | − |
| B21 | 8 | 8 | t16951 | B_III | − | I | + | + | + | + | + | + | + | − | − | + | − | + | − | − | − | + | + |
| B24 | 8 | 8 | t5429 | AA | − | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B32 | 12 | 12 | t16526 | BN | − | IV | + | + | + | + | + | + | + | − | − | − | + | − | − | − | − | − | − |
| D | 71 | 97 | t524 | BN | − | I | − | + | + | + | − | + | − | − | − | − | − | − | − | − | − | − | − |
| G | 71 | 97 | t524 | BN | − | I | − | − | − | + | + | + | − | − | − | + | − | − | − | − | − | − | − |
| I | 71 | 97 | t524 | BN | − | I | − | − | − | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| L | 71 | 97 | t524 | I_I | − | I | − | − | − | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B9 | 71 | 97 | t524 | BN | ++ | I | + | − | − | + | − | + | + | − | − | + | − | − | − | − | − | − | − |
| B28 | 71 | 97 | t524 | BN | + | I | − | − | − | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B29 | 71 | 97 | t524 | BN | − | I | + | − | − | + | + | + | + | − | − | + | − | − | − | − | − | − | − |
| B30 | 71 | 97 | t524 | BN | − | II | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B15 | 4104 | 97 | t524 | BN | − | I | + | − | − | + | − | + | + | − | − | + | − | − | − | − | − | − | − |
| J | 352 | 97 | t521 | AO | − | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B13 | 97 | 97 | t521 | R | − | I | + | + | + | + | − | + | + | − | − | + | − | − | − | − | − | − | − |
| K | 97 | 97 | t13277 | R_I | − | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| M | 97 | 97 | t13277 | R_I | ++ | I | + | + | + | + | − | + | + | − | − | − | − | − | − | − | − | − | − |
| B1 | 97 | 97 | t3782 | ND | − | I | − | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B22 | 97 | 97 | t15786 | R_IV | − | I | + | + | + | + | + | + | + | − | − | + | − | − | − | − | − | − | − |
| B27 | 97 | 97 | t17143 | R_I | − | I | − | + | + | + | − | − | − | − | − | + | − | − | − | − | − | − | − |
| B12 | 3078 | 97 | t359 | R_VI | + | I | + | + | + | + | − | + | + | − | − | + | − | − | − | − | − | − | − |
| B20 | 3078 | 97 | t359 | R_VI | ++ | I | + | + | + | + | − | + | + | − | − | + | − | − | − | − | − | − | − |
| B11 | 4162 | 4162 | t2420 | R_I | − | I | + | + | + | + | − | + | + | − | − | − | − | − | − | − | − | − | − |
| B10 | 130 | 130 | t1773 | ND | +++ | III | + | + | + | + | − | + | − | − | − | − | − | − | − | − | − | − | − |
| B5 | 188 | 188 | t16952 | S | ++ | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| A | 389 | 389 | t164 | F | − | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | + | − | − |
| C | 389 | 389 | t164 | F_I | − | I | + | + | + | − | + | + | − | − | − | − | − | − | + | − | + | − | − |
| N | 389 | 389 | t164 | F | − | I | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B3 | 504 | 504 | t529 | C | − | II | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| B23 | 504 | 504 | t529 | C | − | II | + | + | + | + | + | − | + | − | − | + | − | − | + | − | + | − | − |
| B19 | 522 | 522 | t5428 | AA | ++ | I | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − | − | − |
| B31 | 522 | 522 | t5428 | AA | +++ | III | − | + | + | + | − | + | + | − | − | − | − | − | − | − | − | − | − |

[a] Abbreviations are: ST, sequence type as defined by Multilocus Sequence Typing (MLST); CC, clonal complex as defined by MLST; GT, genotype as defined by RS-PCR; BPI, biofilm production index; Haem, haemolysin. Regulator gene *agr* is *agr* type. All strains tested typed positive for adhesin *fnba* and haemolysin *hlb* and typed negative for MRSA *mecC*, and enterotoxins *seb* and *sec*.

our sample 50% of CC97 isolates (9/18), and 63% of GTR or variants isolates (5/8) were positive for *sed* (Table 4). *seh*, which has been responsible for milk-based SFP outbreaks (Bianchi et al., 2014), was detected in all MRSA ST1-t127 isolates (and in the ST1-t127 MSSA in the same cluster), consistently with the finding of Hummerjohann et al. (2014).

SEA and SED are the SEs most frequently associated with SFP (Hummerjohann et al., 2014; Sabike, Fujikawa, Sakha, & Edris, 2014). 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). In our samples the contamination of raw milk averaged $10^2$ cfu $g^{-1}$, while the average contamination of aged cheese was $10^4$ cfu $g^{-1}$ (data not shown), indicating that in the early phases of cheese-making the *S. aureus* contamination could have reached a concentration critical for the production of SEs ($10^5$ cfu $g^{-1}$; Hummerjohann et al., 2014). Indeed, one of the samples (corresponding to B6 isolate) was referred to our laboratory for the suspect involvement in a SFP episode, and found positive for SEA (data not shown).

### 3.3. Biofilm formation

In food industries, biofilm is an important source of microbial contamination which can lead to spoilage and transmission of foodborne pathogens. In the present work, we studied biofilm forming ability of 49 dairy isolates by using polystyrene microtiter plates as this material is commonly used in food processing industry (Paz-Méndez et al., 2017). The biofilm phenotype was evaluated at 37 °C, which is the optimum growth temperature for *S. aureus* (Vázquez-Sánchez, Habimana, & Holck, 2013). Our results indicated that the isolates had different capability to form biofilm

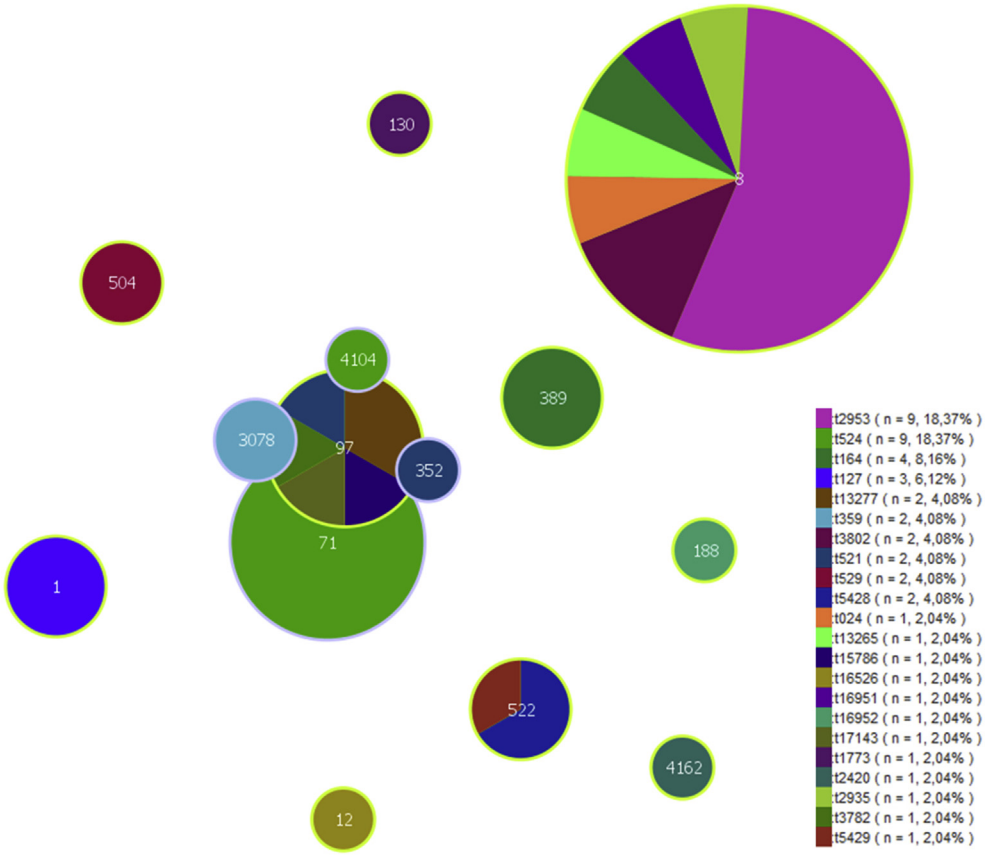


**Fig. 1.** Minimum spanning tree of the 49 *S. aureus* isolates analysed with MLST. The minimum spanning tree was constructed by the goeBURST algorithm using the Phyloviz software v1.1 (http://www.phyloviz.net/). Each circle represents a single sequence type (ST). Yellow outlines indicate group founders, while grey outlines are single locus variants (n-1 loci in common). The colours of the circle filling correspond to the different *spa* types, and the size of the circles is proportional to the number of isolates belonging to the same ST. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 5**
Enterotoxin gene profiles.[a]

| Number of isolates | Enterotoxin gene | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *sea* | *sed* | *see* | *ser* | *seg* | *seh* | *sei* | *sej* | *sep* |
| 1 | | | | | | + | + | | |
| 2 | | | | | | + | | | |
| 3 | | + | | + | | | | + | |
| 1 | + | | | + | | | | + | |
| 1 | | + | | + | | | | | |
| 7 | + | + | | + | | | | + | |
| 1 | | | | + | | | | | |
| 1 | + | | | | | | | | |
| 1 | | + | | + | | | | + | + |
| 1 | | | + | | | | | | |
| 9 | | + | | | | | | | |
| 1 | | | | | | | + | | |
| 1 | | | | | + | | + | | |
| 1 | | + | | | + | | + | | |

[a] No isolates tested were positive for enterotoxin genes *seb* or *sec*.

on the tested hydrophobic surface. Similar observations were also reported by a previous investigation of *S. aureus* isolated from food sector (Di Ciccio et al., 2015). Fig. 2 shows the ability of the 49 dairy isolates and reference strains (ATCC35556, ATCC12600, ATCC12228), to produce biofilms in polystyrene tissue culture plates at 37 °C for 24 h. Out of 49 dairy isolates, 30/49 (61.2%) did not produce biofilm, whereas 19/49 (38.7%) were biofilm producers. Among biofilm producer isolates, 9/19 (47.3%), 8/19 (42.1%) and 2/19, (10.5%) exhibited weak, moderate, or strong biofilm-forming ability, respectively. The highest amount of biofilm was formed by two strains (B10 BPI = 2.192; B31 BPI = 2.538) isolated from cheese and curd, respectively, which were able to show a BPI greater than positive control (ATCC 35556) (Fig. 2; Table 3). Remarkably, 5/19 (26.3%) and 8/19 (42.1%) isolates classified as moderate and weak biofilm producer respectively, also harboured SEs genes (Table 4). The biofilm-forming ability of isolates that are also potential enterotoxins producers should be of concern for food safety, since they may colonise and spread in dairy producing plants and cause contamination of the dairy products (Vergara et al., 2017). The two MRSA ST1-t127-*seh* positive isolates were classified as moderate or weak biofilm producers.

Previous studies showed a correlation between GTB and biofilm formation, with 55% of the biofilm forming isolates belonging to GTB (Thiran et al., 2018). In this work, 40% of the ST8-GTB isolates were biofilm producers, and represent 30% of all biofilm producing isolates (Table 4). Given the wide prevalence of this genotype in the dairy production chain, further studies aimed at investigating the mechanism of biofilm formation specific to ST8-GTB *S. aureus* may be useful to better control the pathogen at herd and plant level.

### 3.4. Genes involved in biofilm production

All isolates were investigated for the presence of nine biofilm associated genes, encoding for adhesins (*icaA*, *icaD*, *cna*, *fnbA*, *fnbB*, *and clfA)*, toxins (*hla*, *hlb*), and regulators (*sarA*). Furthermore, the isolates were genotyped by the *agr* locus. Almost all *S. aureus* isolates were positive for at least five of the biofilm related genes investigated in this study (Tables 4 and 6). The most prevalent

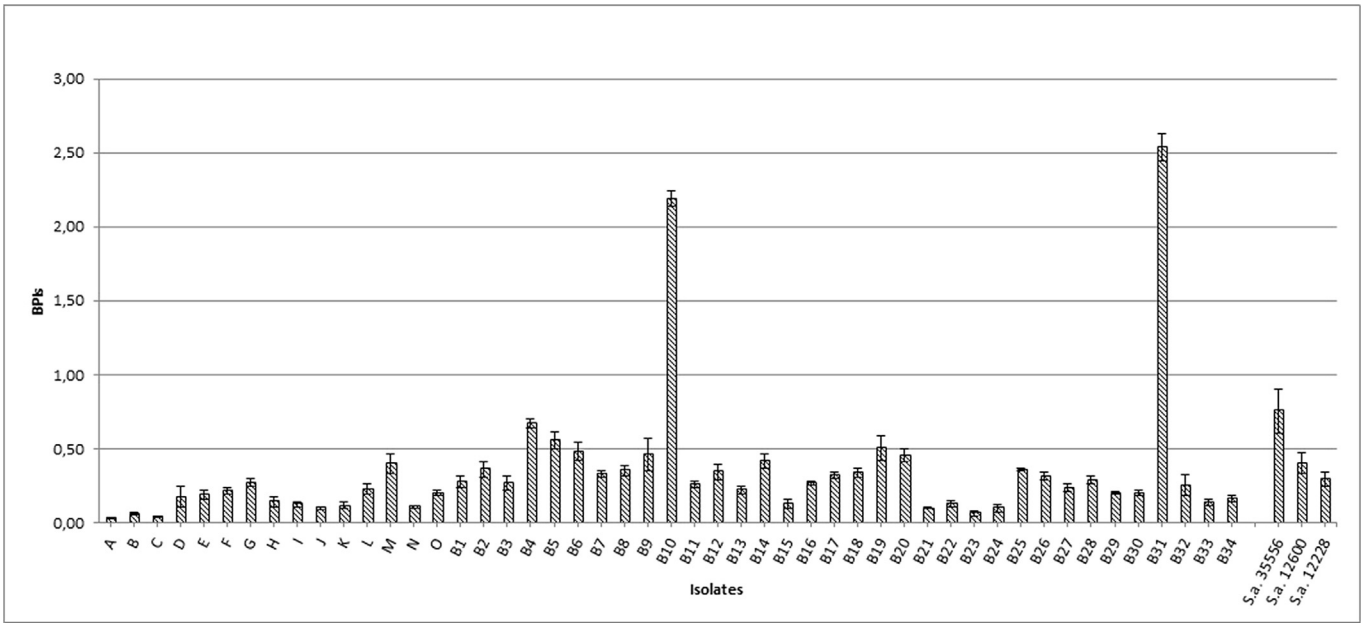


**Fig. 2.** Biofilm production indices of the 49 *S. aureus* isolates and of the three reference strains.

**Table 6**
Patterns of the biofilm related genes and their associations with biofilm production and *agr* type.

| Biofilm related gene patterns | N. of isolates | Biofilm producing | Biofilm negative | *agr* type | | | |
|---|---|---|---|---|---|---|---|
| | | | | I | II | III | IV |
| *sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb* | 21 | 9 | 12 | 16 | 3 | 1 | 1 |
| *sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*clf*A-*hlb* | 5 | 0 | 5 | 5 | – | – | – |
| *sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*hla*-*hlb* | 1 | 0 | 1 | – | 1 | – | – |
| *sar*A-*ica*A-*ica*D-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| *sar*A-*ica*A-*ica*D-*fnb*A-*fnb*B-*clf*A-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| *sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hlb* | 1 | 1 | 0 | – | – | 1 | – |
| *sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*hlb* | 1 | 1 | 0 | 1 | – | – | – |
| *sar*A-*cna*-*fnb*A-*clf*A-*hla*-*hlb* | 2 | 1 | 1 | 2 | – | – | – |
| *sar*A-*cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| *sar*A-*ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hla*-*hlb* | 7 | 5 | 2 | 5 | – | 2 | – |
| *ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hla*-*hlb* | 1 | 1 | 0 | – | – | 1 | – |
| *ica*A-*ica*D-*cna*-*fnb*A-*clf*A-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| *ica*A-*ica*D-*cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| *ica*A-*ica*D-*cna*-*fnb*A-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| *cna*-*fnb*A-*fnb*B-*clf*A-*hla*-*hlb* | 3 | 1 | 2 | 3 | – | – | – |
| *cna*-*fnb*A-*fnb*B-*clf*A-*hlb* | 1 | 0 | 1 | 1 | – | – | – |
| Total | 49 | 19 | 30 | 39 | 4 | 5 | 1 |

genes were *fnbA* gene and *hlb* gene which were detected in all of the isolates. The *icaA* gene was always associated with *icaD* gene, and they were found in 42 (85.7%) isolates. The prevalence rates of *cna*, *clfA sarA*, *hla*, and *fnbB* were 95.9%, 93.9%, 83.7%, 79.2%, and 73.6%, respectively. No correlation was found between the presence of the genes and biofilm production, as also reported in other studies (Tang, Chen, Li, Zeng, & Li, 2013).

*Agr* type I was detected in most of the isolates (39/49; 79.6%), followed by *agr*III (5/49; 10.2%), *agr*II (4/49; 8.2%), and *agr*IV (1/49; 2%). Among the biofilm-negative isolates (n = 30), 25 (83.3%) carried *agr*I, 3 (10%) carried *agr*II and one was *agr*IV. As for the biofilm producing isolates (n = 19), the majority (13, 68.4%) carried *agr*I, 5 (26.3%) carried *agr*III and 1 carried *agr*II. Interestingly, all *agr*III isolates, which were positive for *ica*A, *ica*D, *cna*, *fnb*A, *clf*A, and *hlb* simultaneously, were able to form biofilm, and among them there were the isolates with the highest biofilm production (B10 BPI = 2.192; B31 BPI = 2.538; Fig. 2). These findings suggest an association between this *agr* group and biofilm forming ability, which was also confirmed by the statistical analysis (OR 22.2; CI95% 2.21–2281). Similar results were reported in other studies (Fabres-Klein, Caizer Santos, Contelli Klein, Nunes de Souza, & de Oliveira Barros Ribon, 2015; Khoramrooz et al., 2016). However, these findings are in contrast with other authors that reported *agr*II and *agr*I as involved in higher biofilm production than other *agr* types in *S. aureus* isolated from bovine mastitis (Bardiau, Detilleux, Farnir, Mainil, & Ote, 2014; Cafiso et al., 2007). The *agr* quorum sensing system controls the expression of virulence factors and is also involved in biofilm regulation at structuring and dispersal stages (Boles & Horswill, 2008). It has been observed that repression of *agr* is necessary for biofilm formation, while its activation is essential for the detachment of biofilm (Tan, Li, Jiang, Hu, & Li, 2018). The discrepancies among studies may be related to the lower prevalence of *agr*III in *S. aureus* isolated from bovine mastitis compared with *agr* I and II (Gilot & van Leeuwen, 2004; Melchior et al., 2009), for this reason it may be useful to extend the number of studied isolates to elucidate the results found to date.

## 4. Conclusions

We analysed the clonal diversity of 49 *S. aureus* isolates from the dairy production chain in Northern Italy and determined their virulence gene profile and biofilm formation ability. A diversity of STs was observed among the *S. aureus* isolates, with STs common to multiple dairy facilities identified. The majority of the STs-*spa* types-GTs identified have been reported to be livestock-associated (Cosandey et al., 2016; Feltrin et al., 2015); however, it is not possible to exclude events of contamination originating from humans, due to the finding of genotypes common in the human population (e.g. ST1). Further, should be of concern the capability of some strains, and in particular MRSA, to infect different hosts and become a possible public health threat.

No correlation between presence of genes involved in biofilm formation and biofilm production was found. Based on statistical analysis, the *agr* type III seems to be related biofilm production at the conditions tested in this study. Extending the analysis to a bigger set of isolates could help to substantiate these findings.

The biofilm-forming ability of dairy isolates that encode SEs genes should be of concern, since they may colonise and spread in dairy-producing plants leading to food contamination and causing food safety issues.

Raw milk is commonly used for the production of many traditional cheeses because of the characteristic organoleptic features of the final product. As a consequence, the hygienic condition of the milk used for cheese-making is crucial for the final product quality and safety. Based on our results, the major source of *S. aureus* contamination seems to be the presence of infected animals in the herd. In this perspective, it would be crucial to implement at the farm level actions aimed at minimising the risk of animal infection. However, attention should also be paid to the hygiene of environment, in particular to avoid the persistence of the bacterium in biofilms in cheese manufacturing environments.

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