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Biofilm formation of Staphylococcus aureus dairy isolates representing different genotypes

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ABSTRACT

The objective of this study was to compare the biofilm-forming capabilities of different genotypes of Staphylococcus aureus dairy isolates from Switzerland and northern Italy, including Staph. aureus genotype B (GTB) and methicillin-resistant Staph. aureus (MRSA). We hypothesized that biofilm formation might be more pronounced in the contagious GTB isolates compared with other genotypes affecting individual animals. Twenty-four dairy isolates, including 9 MRSA, were further characterized by genotyping by using ribosomal spacer PCR, spa typing, biofilm formation under static and dynamic conditions, and scanning electron micros- copy. The GTB isolates (n = 6) were more able to form biofilms than other genotypes at 37°C and at 20°C after 48 and 72 h of incubation in the static assay using poly- styrene microtiter plates. This result was supported by scanning electron micrographs showing a GTB isolate producing strong biofilm with extracellular matrix in contrast to a genotype C isolate. Furthermore, none of the MRSA isolates formed strong biofilms in the static assay. However, some MRSA produced low or moder- ate amounts of biofilm depending on the applied condi- tions. Under dynamic conditions, a much more diverse situation was observed. The ability of GTB isolates to be strong biofilm formers was not observed in all cases, emphasizing the importance of growth conditions for the expression of biofilm-related genes. No specific genotype, spa type, or MRSA isolate could be catego- rized significantly into one level of biofilm formation. Nineteen percent of isolates behaved similarly under static and dynamic conditions. The results of this study expand our knowledge of different dairy-related Staph. aureus subtypes and indicate the benefit of genotyping when biofilms are studied.

INTRODUCTION

Staphylococcus aureus is a foodborne pathogen con- sidered the third most important causative bacterial agent of foodborne illnesses worldwide (Hennekinne et al., 2012); it is of great concern to the dairy industry (De Buyser et al., 2001; Oliver et al., 2009). In particu- lar, dairy cow mastitis is the most important disease in the global dairy industry and Staph. aureus is one of the most important etiological agents of contagious mastitis (Silva et al., 2013; Voelk et al., 2014). An- other major concern is that Staph. aureus can form biofilms (Santos et al., 2014). Biofilms are aggregates of microbial cells surrounded by a matrix of exopoly- mers (Costerton et al., 1999). Besides the production of exotoxins and surface proteins, the formation of these highly organized multicellular complexes is increasingly recognized as an important virulence factor in Staph. aureus (Tang et al., 2012; Lee et al., 2014). Biofilm formation can lead to persistent contamination or infec- tion because the cells within the biofilm are very resis- tant to sanitation procedures and to the action of the host immune system and antimicrobial agents (Song et al., 2016). Different sources of Staph. aureus in the dairy cow environment have been described (Zadoks et al., 2002). Infected animals (cow-to-cow transmission), workers, and equipment and utensils used for milking are the main sources of the microorganism (Lee et al., 2014). Although some researchers have studied the abil- ity of members of the Staphylococcus genus to adhere to surfaces and form biofilm, most studies have addressed the clinical aspects related to biofilm formation by Staphylococcus intermedius on medical implants and materials (Donlan and Costerton, 2002; de Souza et al., 2014). Moreover, few studies have reported biofilm formation by Staph. aureus isolated from ready-to-eat- foods (Oniciuc et al., 2016). Additionally, recent studies have identified several genotypes of Staph. aureus

that differ in their contagiosity and pathogenicity (Fournier et al., 2008; Voelk et al., 2014; Cosandey et al., 2016). Graber et al. (2009) further demonstrated that geno- type was highly associated with virulence gene pattern. Among different genotypes, Staph. aureus genotype B (GTB) is associated with high within-herd prevalence, indicating an increased contagious and virulence po- tential compared with other genotypes (Graber et al., 2009; Voelk et al., 2014). In particular, Staph. aureus GTB, a major contaminant in Swiss raw milk cheese (Hummerjohann et al., 2014), was characterized by the presence of the enterotoxin genes sea, sed, and sej, and a SNP lukE gene (lukEB; Cosandey et al., 2016). Genotype B has been found not only in Switzerland, but also in other countries of central Europe, including Italy, indicating that it is a relevant international prob- lem in cow milk production (Cosandey et al., 2016). Regarding these aspects, the current study was carried out to compare the biofilm-forming capabilities of dif- ferent genotypes of Staph. aureus dairy isolates, includ- ing Staph. aureus GTB and methicillin-resistant Staph. aureus (MRSA), because MRSA are a severe problem in the human population and have been isolated from milk, cheese, and other foodstuffs in different countries (Normanno et al., 2007; De Boer et al., 2009; Kav et al., 2011). We evaluated the ability of Staph. aureus dairy isolates to form biofilm under static and dynamic conditions and by using scanning electron microscopy. We hypothesized that biofilm formation might be more prevalent in the more contagious GTB strains com- pared with other genotypes (OGT).

MATERIALS AND METHODS

Bacterial Isolates

The experiment was conducted on 24 isolates (in- cluding 6 GTB strains) from milk and milk products. One isolate from poultry meat (PR 281), previously described as strong biofilm producer (Di Ciccio et al., 2015), and 3 strains from a culture collection (ATCC3556, ATCC12600, ATCC12228; American Type Culture Collection, Manassas, VA) were included as reference strains (Table 1). Stock cultures were stored at −80°C, and strains were incubated for 24 h at 37°C in tryptic soy broth (TSB, BBL Becton Dickinson, Le Pont de Claix, France) before experiments.

Extraction of Nucleic Acids

A single colony of Staph. aureus was resuspended in 100 µL of Tris-EDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), incubated at 95°C for 10 min, and immediately placed into ice. For PCR analysis, the lysate was diluted 1:100 in H2O and directly used for amplification.

Genotyping

Genotyping of the strains was based on PCR ampli- fication of the 16S-23S rRNA intergenic spacer region (RS-PCR) and was performed as described by Fournier et al. (2008). Briefly, the PCR reaction mix (total vol- ume of 25 µL) contained 1× HotStarTaq Master Mix (Qiagen AG, Hombrechtikon, Switzerland), 800 nmol of each primer G1 and L1 (Jensen et al., 1993), and 30 µg of the lysate nucleic acids. The PCR conditions were as follows: denaturation at 95°C for 15 min followed by 27 cycles of 94°C for 1 min, 2-min ramp time, annealing at 55°C for 7 min, 2-min ramp time, and extension for at 72°C for 2 min on a T-Professional thermal cycler (Biometra, Göttingen, Germany). The PCR products were analyzed by the miniaturized electrophoresis sys- tem DNA 7500 LabChip (Agilent Technologies, Basel, Switzerland). The resulting amplification patterns were interpreted according to Fournier et al. (2008), using a computer program developed in-house (Syring et al., 2012).

spa Typing

The spa typing was based on the amplification of the spacer region of the spa gene of Staph. aureus whichencodes staphylococcal protein A. It was performed ac- cording to the method described by Boss et al. (2016). Briefly, the PCR reaction mix (total volume of 25 μL) contained 300 nmol of each primer, 12.5 μL of of HotStarTaq Master Mix (Qiagen AG), and 2.5 μL of template DNA. The PCR cycles included a denatur- ation step at 95°C for 15 min, followed by 37 cycles of denaturation at 94°C for 60 s, annealing at 6The 0°C for 60 s, and a single extension step at 72°C for 10 min on a T-Professional thermal cycler (Biometra). PCR products were sent to Microsynth AG (Balgach, Switzerland) for purification and sequencing using the Sanger approach. The obtained sequences were then evaluated for corresponding spa type (t) using the Ri- dom server (http://www.spaserver.ridom.de/).

Detection of nuc and mecA Genes

All isolates were confirmed as MRSA by the detec- tion of the methicillin resistance mecA gene and ther- mostable nuclease nuc gene. The DNA extracts were subjected to a duplex-PCR protocol for the detection of mecA and nuc (Virgin et al., 2009). A methicillin-susceptible Staph. aureus strain (ATCC29213) was used as a negative control and a MRSA strain (ATCC33591) as a positive control.

Biofilm Formation Under Static Conditions

All strains were tested in triplicate on polystyrene tissue culture plates at different temperatures (37°C, 20°C) and incubation times (24, 48, and 72 h) for biofilm production. For this purpose, 2 Staph. aureus and the Staph. epidermidis reference strains were used as control to define different categories of the Staph. aureus isolates to be studied. Biofilm formation, ex- pressed as biofilm production index (BPI), was com- pared with reference strains: Staph. aureus ATCC35556 (strong biofilm producer; Cramton et al., 1999; Seidl et al., 2008) as positive control (BPIPC); Staph. aureus ATCC12600 (moderate biofilm producer; Di Ciccio et al., 2015) (BPI12600); Staph. epidermidis 12228 (nega- tive biofilm producer; Atshan et al., 2012; Lee et al., 2014) as negative control (BPINC) for each isolate (Ta- ble 1). The cutoff point for biofilm production was the BPI value obtained by BPINC on polystyrene (0.294). Staphylococcus aureus strains showing the ability to pro- duce biofilms were classified as weak (BPINC ≤ Staph. aureus BPI < BPI12600), moderate (BPI12600 ≤ Staph. aureus BPI < BPIPC), or strong (Staph. aureus BPI ≥ BPIPC). Before conducting the experiments, Staph. aureus strains were activated by culturing twice in 10 mL of TSB (Oxoid S.p.A., Milan, Italy) at 37°C for 24 h following a previously described method (Di Ciccio et al., 2015). Cultures of Staph. aureus, from overnight tryptic soy agar (Oxoid) growth, were prepared in TSB by incubating at 37°C. Cultures were then washed 3 times with PBS (pH 7.3, Sigma-Aldrich S.r.l., Milan, Italy) and diluted with fresh TSB to reach a concen- tration of about 108 cfu/mL, which was assessed by reading the optical density (OD) at 550 nm using a Varian SII Scan Cary 100 spectrophotometer (Agilent Technologies, Santa Clara, CA). Three milliliters of the standardized inoculum was then added to polystyrene tissue culture plates (961 mm2, 35 mm in diameter). Samples were then incubated at 37°C (for 24 h) and 20°C (for 48 or 72 h). After incubation, nonadherent 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 destain 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 destaining solution was measured at 492 nm (Victor, Perkin Elmer, Waltham, MA). All results were expressed by calculating the BPI as follows: BPI = [ODmean biofilm surface (mm2)−1]

× 1,000. Biofilm formation, expressed as BPI, was compared with refer- ence strains for each isolate. Finally, all isolates were assigned to different categories based on their BPI values.

Biofilm Formation Under Dynamic Conditions

Biofilm formation under flow conditions was evalu- ated on 24 dairy isolates and 4 reference strains using a BioFlux 1000 microfluidic system (Fluxion Biosci- ences Inc., San Francisco, CA) as previously described (Moormeier et al., 2013) with some modifications. This device enables accurate control of fluid flow and per- mits simultaneous growth of multiple biofilms (Benoit et al., 2010). To grow biofilms in the BioFlux device, 48-well plates (Fluxion Biosciences Inc.) were used. The microfluidic channels (70 × 370 μm) were primed for 2 min with 200 μL of TSB at 2.0 dyn/cm2 (where 1 dyn = 10−5 N). After priming, the TSB was carefully removed from the outlet wells and replaced with 20 μL of fresh overnight culture of Staph. aureus adjusted to reach an inoculum concentration of 108 cfu/mL. The channels were seeded by pumping from the outlet wells to the inlet wells at 2.0 dyn/cm2 for 5 s. Cells were then allowed to attach to the surface of the channels for 1 h at 37°C. Excess inoculum was aspirated from the outlet wells, and 1.2 mL of TSB was added to the inlet wells and pumped at 0.6 dyn/cm−2 for 17 h. For each isolate tested, one bright-field image per channel was acquired at 5-fold magnification in 30-min intervals for a total of 35 time points (17 h). Images were always taken at the middle of the channel (channel numbers and arrows on the plate were used as landmarks) with a digital camera, and gain, exposure, and magnifica- tion were kept constant for all images. Every isolate was tested in biological triplicates with 2 channels for each replicate. Biofilm-forming ability was evaluated by classifying isolates into 3 main phenotypical catego- ries: biofilm, bacterial accumulation/aggregates, and non-biofilm. The biofilm phenotype included isolates forming dense, stable aggregates of bacteria sticking to the surface of the channel over time. Bacteria forming small, diffuse aggregates or bacterial smear were classified in the bacterial accumulation/aggregates phenotype. Finally, the non-biofilm phenotype includes all isolates presenting adherent bacteria only, with no accumulation abilities or forming unstable aggregates. All phenotypic observations were made on the entire time-lapse movie. The observation of a stable biofilm structure at least once during the time course of the experiment automatically classified the isolate in the biofilm-forming category. Figure 1 illustrates the dif- ferent phenotypes, and Figure 2 summarizes the clas- sification method. The 6 replicates of each isolate were classified into 1 of the 3 phenotypic categories. For each isolate, the total number of replicates belonging to each phenotype was calculated. Based on this, a first distinction was made regarding the isolates' behavior in terms of biofilm formation under flow conditions. Isolates were subcategorized into 3 groups: (1) the consistent group, including isolates presenting the same phenotype for each replicate; (2) the dominant group, including isolates presenting a dominant phenotype ob- served more often than the others among the replicates, and (3) the inconsistent group, including isolates that randomly showed different phenotypes. The isolates belonging to the consistent and dominant groups were further classified according to their biofilm formation potential following the method described before. For the dominant group, the dominant phenotype was cho- sen. This classification method gives information about biofilm formation phenotype and its reproducibility.

Scanning Electron Microscopy of Staph. aureus Biofilm

Biofilm formation was further confirmed by scanning electron microscopy. Two isolates were selected: Staphaureus 18 (GTB) and Staph. aureus 13 (genotype C, GTC), and biofilms were prepared as described above. The microbial cells were grown at 37°C for 24 h on polystyrene tissue plates and then washed by dipping 3 times in sterile PBS to remove nonadherent cells. Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate

buffer (pH 7.2) for 30 min at room tem- perature and then fixed in 1% osmium tetroxide (for 1 h). Samples were then washed with 0.1 M cacodylate buffer for 1 h to remove any unreacted glutaraldehyde before rinsing and dehydration. Samples were dehy- drated through a series of alcohols and dried to critical point with liquid CO2 (CPD 030 Baltec, Leica Micro- systems GmbH, Wetzlar, Germany). Specimens were then sputter-coated with a gold-palladium layer using a SCD 040 coating device (Balzer Union, Liechtenstein). Samples were observed using a Zeiss DSM 950 scanning electron microscope at an accelerating voltage of 10 kV (Zeiss, Oberkochen, Germany). The images were processed for display using Photoshop (Adobe Systems Inc., San Jose, CA).

Statistics

All experiments were carried out in triplicate and repeated in 2 independent sets of experiments. Data are shown as mean ± standard deviation (SD), and IBM SPSS Statistics 23 (IBM Corp., Armonk, NY) was used for statistical analysis. The significance of differences in biofilm formation between GTB group and OGT group was assessed by one-way ANOVA, followed by Scheffé test. Differences were considered significant when $P < 0.05$.

RESULTS

Genotyping and spa Typing

The Staph. aureus dairy isolates of this study were genotyped and the results are summarized in Table 2. The RS-PCR analysis revealed 11 different genotypes with 3 genotypes carrying variants, and 18 spa types were detected, including a new one. Furthermore, 9 of the chosen isolates (7 strains from bovine milk, 1 strain from goat milk, 1 strain from sheep milk) carried the mecA gene and were thus categorized as MRSA (Table 2).

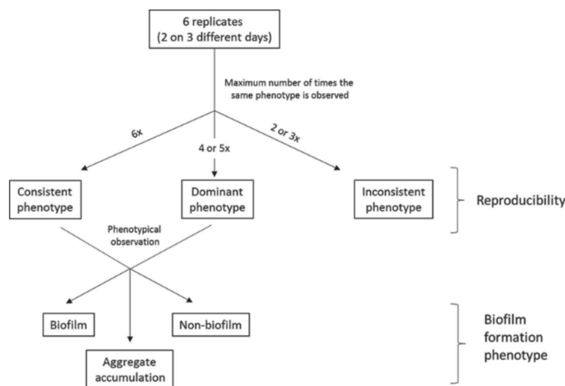
Biofilm Formation in the Static Model

Differences in biofilm formation were observed be- tween the Staph. aureus isolates tested. Figure 3 shows the ability of the 24 Staph. aureus dairy isolates and ref- erence strains (ATCC35556, ATCC12600, ATCC12228, PR 281), to produce biofilms in polystyrene tissue cul- ture plates. Results are summarized in Table 3.

At 37°C (24 h), out of 24 dairy isolates, 13 (54%) did not produce biofilm, whereas 11 (45.8%) were classified as weak (n = 2), moderate (n = 8), or strong (n =
1) biofilm producers, respectively. It was shown that none of the biofilm-negative strains were GTB. Among biofilm-positive strains, 6 (54.5%) GTB isolates had the ability to form moderate (n = 5) or strong (n = 1, isolate no. 18) biofilm, whereas 3 (25%) MRSA isolates had the ability to form moderate (n = 2) or weak (n =
1) biofilm.

At 20°C (48 h), out of 24 Staph. aureus dairy isolates, 17 (70.8%) strains did not produce biofilm, whereas 7 (29.2%) were classified as weak (n = 2), moderate (n
= 4), or strong (n = 1) biofilm producers, respectively. It was shown that none of the biofilm-negative (17) strains were GTB. Among the biofilm-positive strains, 6 (85.7%) GTB isolates had the ability to form weak (n= 1), moderate (n = 4), or strong (n = 1, isolate no. 19) biofilm, whereas 1 MRSA isolate (no. 1140) was a weak biofilm producer.

At 20°C (72 h), out of 24 Staph. aureus dairy isolates, 17 (70.8%) strains did not produce biofilm, whereas 7 (29.2%) strains were classified as weak (n = 2), mod- erate (n = 4), or strong (n = 1) biofilm producers. Only one MRSA strain (no. 1140) was classified as a



weak biofilm producer. It was shown that none of the biofilm-negative strains (17) were GTB. Among the 7 biofilm-positive strains, 6 (85.7%) isolates belonging to GTB had the ability to form weak (n = 1), moderate (n= 4), or strong (n = 1, isolate no. 27) biofilm, respec- tively. Only 1 non-GTB isolate (no. 1140, MRSA) was biofilm positive (weak biofilm producer) at 20°C (48 to 72 h), although it was negative at 37°C (24 h). Interest- ingly, the dairy isolate Staph. aureus 18 (GTB strain) showed a higher BPI value (at 37°C on polystyrene) than the poultry isolate PR 281 (no GTB strain, spa type t002, non-MRSA) that was classified as a strong biofilm producer. Finally, none of the MRSA isolates (n= 9) was classified as a strong biofilm producer. Correlations between GTB strains and biofilm for- mation were detected by statistical analysis. The GTB strains were statistically more able ($P < 0.05$) to form biofilm than OGT at 37°C and 20°C (at 48 and 72 h of incubation).

Scanning Electron Microscopy Analysis of Staph. aureus Biofilm

Two Staph. aureus genotypes were selected for scanning electron microscopy based on their different biofilm formation on polystyrene. In particular, Staph. aureus no. 18 (GTB) showed a BPI value higher than the strong biofilm producer, PR281 (Di Ciccio et al., 2015), whereas Staph. aureus no. 13 (GTC) showed a weak biofilm-producing ability. Representative mi- crographs of biofilms produced by 2 analyzed isolates are shown in Figure 4. In particular, one micrograph showed Staph. aureus no. 13 forming a rudimentary biofilm consisting of sparse aggregates of cells bound by few or absent extracellular polymeric substances (Fig- ure 4 a,b). In contrast, Staph. aureus no. 18 showed a complex 3-dimensional meshwork-like structure of cells at high density, embedded in a network of extracellular polymeric substances (Figure 4 c,d).

Biofilm Formation in the Dynamic Model

Isolates were first classified according to the repro- ducibility of their biofilm formation behavior under flow conditions using the BioFlux device. Out of the 28 tested isolates (24 dairy isolates, 1 poultry isolate, and 3 reference strains), 5 (17.9%) show a consistent phenotype: all 6 replicates of each isolate displayed the same phenotype. Sixteen (57.19%) of them displayed a dominant phenotype, with 4 or 5 replicates out of 6 presenting the same phenotype. Finally, for 7 (25%) of the isolates, including 2 MRSA, the phenotypes observed were not consistent from one replicate to an- other. Therefore, no biofilm phenotype was attributed to those isolates. The 21 (75%) isolates belonging to the consistent and dominant groups were further character- ized for their biofilm-forming potential. Of them, 14 isolates (66.7%), including 5 MRSA, presented biofilm structures, whereas only 3 (14.3%) showed the diffuse aggregate/accumulation phenotype and 3

(19.1%), including 1 MRSA, were unable to accumulate and remained as adherent bacteria (Table 4).

Out of the 6 GTB isolates, 3 were non-biofilm- formers (no. 19, 25, 27), 2 were biofilm formers (no. 18 and 30), and 1 could not be classified according to its biofilm-forming behavior because of inconsistency in reproducibility (no. 22).

Comparison of Biofilm Formation Between Static and Dynamic Conditions

The biofilm-forming potential of isolates was com- pared between static and dynamic conditions at 37°C (Table 5). Of 25 isolates, comparisons were performed for the 21 for which a phenotype could be attributed under dynamic conditions. Because the other 4 were unable to form reproducible structures under dynamic conditions, they were not included in the comparison.

Categorization under static conditions was based on 4 categories, whereas isolates were classified into 3 groups under dynamic conditions. We assumed that weak and moderate formation potential under static conditions could be compared with the aggregate phenotype ob- served under dynamic conditions. Four isolates (19.1%) displayed similar biofilm-forming potential under both static and dynamic conditions. One was a non-biofilm- former, 1 was a weak-to-moderate biofilm former, and

2 were strong biofilm formers. The majority of the isolates (66.7%) tended to form more biofilm under dy- namic conditions, and 6 of the MRSA belong to these category. Finally, 3 isolates (14.3%) formed less biofilm under dynamic conditions compared with static condi- tions.

DISCUSSION

This is the first study on biofilm formation of Staph. aureus GTB compared with other genotypes of this species, including MRSA isolated from milk and milk products. Staphylococcus aureus isolated from bovine mastitis and cow milk is a genetically heterogeneous group (Cosandey et al., 2016). Among different geno- types, Staph. aureus GTB was found to be associated with high within-herd prevalence, indicating increased contagious and virulence potential compared with other genotypes (Graber et al., 2009; Voelk et al., 2014; van den Borne et al., 2017) and it has been described as a major contaminant in Swiss raw milk cheeses (Hum- merjohann et al., 2014).

Regarding these aspects, this study was carried out with the aim of evaluating the ability of Staph. aureus isolated from milk and milk products to form biofilm under static and dynamic conditions. Additionally, our aim was to test whether Staph. aureus GTB isolates were more likely to be biofilm producers than other Staph. aureus genotypes. The results of biofilm forma- tion in the static model support this hypothesis, as GTB isolates produced significantly more biofilms than other genotypes under the several conditions tested. Further- more, 1 GTB isolate was seen to be a better biofilm producer than GTC in the scanning electron microscopy analysis. Interestingly, the dairy isolate Staph. aureus 18 (GTB strain) showed a higher BPI value than the poultry isolate Staph. aureus PR 281 (not GTB), that was described as a strong biofilm producer by Di Ciccio et al. (2015). Further studies are needed to evaluate the contribution of biofilm formation to the persistence of Staph. aureus GTB in dairy, which has been observed for cheese-making facilities with duration of up to 27 wk (Hummerjohann et al., 2014).

Although the study presented here is one of the few reports on Staph. aureus spa types and biofilm forma- tion in a dairy environment, it is generally believed that strong biofilm formation is linked to certain genetic lineages, as found by several clinical studies (Croes et al., 2009; Naicker et al., 2016). Application of methods on the genomics, transcriptomics, and

proteomics level of those different lineages could probably explain these observed phenotypes in the future.
Furthermore, because not all subtypes of Staph. au- reus are distributed equally all over the world, application of subtyping is an important tool for local dairies. Veh et al. (2015) were able to characterize genotypic and phenotypic Staph. aureus causing persistent and nonpersistent subclinical bovine IMI in Canada. That study, where no GTB was detected, reported that t529 and t267 were the subtypes with the lowest biofilm production, which was confirmed by our study. This in- dicates the need for further regional studies on biofilm formation, including those genotypes that predominate in the milk production of certain specific regions.
Regarding the typing of our isolates, genotypes B, C, F, I, and R, are typically associated with bovine isolates and are the more frequently encountered genotypes when typing European Staph. aureus isolated from cow milk (Cosandey et al., 2016). In general, no correlation between genotype and spa type could be established except for spa type t529, which seemed to be associated with GTC, and t2953, which is the most frequently ob- served spa type of Staph. aureus GTB (Hummerjohann et al., 2014; Boss et al., 2016). In contrast, spa type t524 has been associated with 3 different genotypes. Several spa types described in this study (t524, t127, t267, t529, t204, t295) have previously been associated with bovine isolates in other countries (Hasman et al., 2010; Hwang et al., 2010; Sakwinska et al., 2011; Mitra et al., 2013; Boss et al., 2016).
Another aspect to note in our study was the presence of MRSA among our dairy isolates. Regarding their corresponding spa types, 2 isolates (t127 and t174) were of human origin (Grundmann et al., 2010; Lozano et al., 2011). Many reports have identified MRSA in bovine mastitis cases or in dairy products in several countries including Italy (Normanno et al., 2007; Kav et al., 2011; Kamal et al., 2013). In our study, 9 dairy isolates were classified as MRSA. Among them, 55.5% form biofilms under dynamic conditions and 44.4% displayed weak to moderate biofilm-forming abilities under static condi- tions. Although the exact mechanisms and process of biofilm formation in MRSA are poorly understood, 2 studies performed by the same research group suggest- ed that penicillin binding protein 2a (PBP2a) is also an important factor in biofilm accumulation (Pozzi et al., 2012; Rudkin et al., 2012). Other studies looked at dairy MRSA isolates and highlighted their high biofilm- forming potential. Bardiau et al. (2013) found that all MRSA isolated from bovine mastitis in Belgium were biofilm formers. Prenafeta et al. (2014) described a 50% prevalence of biofilm formation among MRSA isolated in bulk tank milk in Great Britain. It is well known that MRSA detected from milk and dairy products can be staphylococcal enterotoxin producers (Normanno et al., 2007; Parisi et al., 2016). To date, the contribu- tion of the contaminated environment to the spread of antimicrobial resistant microorganisms is not well understood. However, the biofilm-forming ability of MRSA that are potentially staphylococcal enterotoxin producers should be of concern for food safety, because they may colonize and spread in the dairy industry environment, leading to food contamination. Despite the importance of the ica gene locus in biofilm develop- ment, biofilms can occur in an ica-independent fashion. In a preliminary study, we discovered the presence of the icaA gene in some of the genomes of different genotypes. However, as the presence or absence of this gene is not correlated with a certain biofilm formation phenotype (H. U. Graber, unpublished data), further study on expression of the different genes is needed.
With regard to this, ica-independent biofilms appear to be the most important bacterial films produced by MRSA isolates (Vasudevan et al., 2003; Cucarella et al., 2004; O'Neill et al., 2007). The ability of dairy- isolated multidrug resistant bacteria to form biofilms in food processing facilities is of great concern for food safety. First, it contributes to the spread of antibiotic resistance along the food chain. Because biofilms confer an intrinsic resistance to disinfection methods, they are very difficult to eliminate and contribute to bacterial

persistence in food processing facilities. Moreover, the proximity of cells within biofilms favors horizontal gene transfer and risk for resistance transmission to patho- genic bacteria, leading to potential further treatment failure (Verraes et al., 2013).
When looking at the results of biofilm formation under dynamic conditions, the pronounced biofilm- forming potential of Staph. aureus GTB was not that obvious. Indeed, only half of the GTB isolates show biofilm structures and it was impossible to assign a biofilm formation potential for 1 isolate because of in- consistency in the reproducibility. However, the strong biofilm formation potential of Staph. aureus GTB iso- lates under static conditions reveals that this genotype possesses the genetic information necessary to form biofilms under certain conditions.
Only one strain (no. 1140, MRSA) was biofilm posi- tive (weak biofilm producer) at 20°C (48 to 72 h), al- though it was negative for biofilm formation at 37°C (24 h). Rode et al. (2007), in contrast, noted the highest attachment capacity in Staph. aureus on polystyrene at suboptimal growth temperatures (20, 25, and 30°C).
Comparison of our data with reports from Pagedar et al. (2010), da Silva Meira et al. (2012), Lee at al. (2014) and Di Ciccio et al. (2015) on Staph. aureus dairy and other food isolates is rather limited because of the ap- plication of different methods of subtyping (if applied at all) and measurement or categorization of biofilm formation. The comparison between biofilm formation under static and dynamic conditions is even more com- plicated. In clinical isolates, only 19% of the isolates behaved similarly under both static and dynamic con- ditions (Vanhommerig et al., 2014), which is similar to our results (Table 5). Factors including incubation time, growth surface, and nutrients are thought to influence biofilm formation in staphylococci measured by static or dynamic model assays (Stepanović et al., 2001; Vanhommerig et al., 2014; Van Kerckhoven et al., 2016). Furthermore, we have recently shown similar results on biofilm formation of E. coli dairy isolates, noting a lack of correlation between static and dynamic conditions (Marti et al., 2017). From our results, we cannot conclude that differences observed in terms of biofilm formation are due only to the growth under flow conditions because the growth surface was not the same (polystyrene for the static model; glass in the dynamic model), which can influence the adhesion process. Moreover, growth time was slightly different, with a longer incubation time under static conditions.
Finally, the evaluation process differed and could influence the conclusions. For the dynamic conditions, we had a time-lapse overview, whereas biofilm forma- tion under static conditions was evaluated based on an end point state. Despite these differences, the fact that biofilm formation was observed in either condition indicates that the isolate possesses the genetic informa- tion necessary for biofilm formation. Further studies are needed to characterize the underlying mechanism of these phenotypes and to evaluate which of the different assays used for the measurement of biofilm formation best mimics the “real” situation in the dairy environ- ment. Scanning electronic microscopy could be used as a semiquantitative technique, because it allows the observation of bacteria–surface interactions. Thus, in this study, scanning electron microscopy was used to con- firm the biofilm formation of 2 differently categorized strains, and the images confirmed the results obtained from static biofilm assay, showing a strong difference in biofilm formation of 2 different genotypes.

CONCLUSIONS

All Staph. aureus GTB dairy isolates used in the pres- ent study showed significantly higher biofilm formation on polystyrene when exposed to different environmental conditions compared with most of the other genotypes, including MRSA. Under dynamic conditions, the abil- ity of GTB strains to be good biofilm formers was not observed in all cases,

emphasizing the importance of growth conditions for the expression of biofilm-related genes. In summary, the results of this study expand our knowledge of different Staph. aureus subtypes from the dairy field and show the benefit of genotyping when biofilm formation is studied.

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