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INTERPRETIVE SUMMARY

COMMUNICATIONS OF *AUREUS* AND NON-*AUREUS* STAPHYLOCOCCI

MAHMMOD

The role of NAS on the risk of acquisition of *S. aureus* IMI is debated. We investigated the distribution patterns of NAS species from milk and teat skin in dairy herds with automatic milking systems. Additionally we examined if the isolated NAS influence the virulence expression of *S. aureus*. *S. epidermidis* and *S. chromogenes* were milk-associated, while *S. equorum* and *S. cohnii* were teat-associated. *S. chromogenes* and *S. xylosus* showed protective effect against *S. aureus*, while *S. epidermidis* and *S. equorum* showed varied effect based on habitat type and herd-associated factors. *S. Sciuri* and *S. vitulinus* showed no effect.

Communications of *Staphylococcus Aureus* and Non-*Aureus* Staphylococcus species from Bovine Intramammary Infections and Teat Apex Colonization

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ABSTRACT

The role of non-aureus Staphylococci (**NAS**) on the risk of acquisition of intramammary infections (**IMI**) with *Staphylococcus aureus* (***S. aureus***) is vague and still under debate. The objectives of this study were to (1) investigate the distribution patterns of NAS species from milk and teat skin in dairy herds with automatic milking systems (**AMS**), and (2) examine if the isolated NAS influences the expression of *S. aureus* virulence factors controlled by the accessory gene regulator (**a*gr***) quorum sensing system. In eight herds, 14-20 cows with elevated somatic cell count were randomly selected for teat skin swabbing and aseptic quarter foremilk samples from right hind and left front quarters. Teat skin swabs were collected using the modified wet-dry method and milk samples were taken aseptically for bacterial culture. Colonies from quarters with suspicion of having NAS in milk and/or teat skin samples were subjected to MALDI-TOF assay for species identification. To investigate the interaction between *S. aureus* and NAS, 81 isolates NAS were subjected to a qualitative beta-galactosidase reporter plate assay.

In total, 373 NAS isolates were identified representing 105 from milk and 268 from teat skin of 284 quarters (= 142 cows). Sixteen different NAS species were identified, 15 species from teat skin and 10 species from milk. The most prevalent NAS species identified from milk were *S. epidermidis* (50%), *S. haemolyticus* (15%), and *S. chromogenes* (11%) accounting for 76%. Meanwhile, the most prevalent NAS species from teat skin were *S. equorum* (43%), *S. haemolyticus* (16%), and *S. cohnii* (14%) accounting for 73%. Using reporter gene fusions monitoring transcriptional activity of key virulence factors and regulators, we found that out of 81 supernatants of NAS isolates; 77% reduced expression of *hla,* encoding a-hemolysin, 70% reduced expression of RNA-III, the key effector molecule of *agr,* and 61% reduced expression of *spa* encoding Protein A of *S. aureus*, respectively.

Our NAS isolates showed three main patterns; (a) downregulation effect such as *S. chromogenes* (milk) and *S. xylosus* (milk and teat), (b) no effect such as *S. sciuri* (teat) and *S. vitulinus* (teat), and the third pattern (c) variable effect such as *S. epidermidis* (milk and teat) and *S. equorum* (milk and

69 teat). The pattern of cross-talk between NAS species and *S. aureus* virulence genes varied according
70 to the involved NAS species, habitat type, and herd factors. The knowledge of how NAS influences
71 *S. aureus* virulence factors expression could explain the varying protective effect of NAS on *S.*
72 *aureus* IMI.
73
74 **Keywords:** non-*aureus* staphylococci; *Staphylococcus aureus*; microbial interactions; bovine
75 mastitis; automatic milking system; protective effect

INTRODUCTION

Nowadays, non-aureus staphylococci (**NAS**) are the most common cause of bovine intramammary infections (**IMI**) in dairy herds worldwide (Braem et al., 2013; Souza et al., 2016). When studying NAS, aggregating NAS as a group without accurate species identification is no longer recommended, as species-specific differences in behavior, epidemiology, ecology, and impact on udder health have been revealed (Vanderhaeghen et al., 2014). Furthermore, NAS species showed great differences in antimicrobial susceptibility and virulence factors (Sawant et al., 2009). Condas et al. (2017) concluded that considering NAS as a single group has undoubtedly contributed to apparent discrepancies among studies as to their distribution and importance in IMI.

Previous studies have extensively investigated the epidemiological characteristics of NAS for dairy herds with conventional milking systems. However, knowledge about these characteristics or patterns is sparse for dairy cows in automatic milking systems (**AMS**) (Supré et al., 2011; De Visscher et al., 2014). Management of udder health in conventional milking systems differs from AMS (Dohmen et al., 2010; Hovinen and Pyörälä 2011). Cows in AMS can be milked up to 5 times daily without any human contact with the udder. The longer milking duration and exposure of the teat skin to disinfectants may affect the teat skin microbiota. Furthermore, there is high risk for teat colonization and subsequently IMI because up to 60 cows are milked several times daily with the same robot (Rasmussen, 2006).

The epidemiological and ecological characteristics NAS isolated from milk and surrounding environment of cows differ and are associated with the identified species. Results from research studies on NAS are sometimes conflicting. Vanderhaeghen et al. (2015) reported that *S. chromogenes* is a bovine-adapted species involved in many cases of IMI, and *S. simulans* typically causes contagious IMI, while *S. xylosus* appears to be a versatile species. NAS species originating from distinct habitats showed clear differences that may be related to their diversity in ecology and epidemiological behavior (Souza et al., 2016). These different and contradictory results about NAS

characteristics may likely be due to the lack of knowledge about their ecology and epidemiology within and between species (Fry et al., 2014). Therefore, extra efforts are crucial to improve our knowledge on different traits of NAS at the species level in the different habitats for boosting our understanding to their epidemiology in dairy herd context.

The effects of NAS on the risk of acquiring *Staphylococcus aureus* (***S. aureus***) IMI have yielded ongoing debate (Reyher et al., 2012; Vanderhaeghen et al., 2014). Using traditional antibiotics is the most common approach for treatment of *S. aureus* infections and bovine mastitis in general. However, this approach is associated with adverse consequences including emergence of bacterial resistance and antimicrobial residues in milk (Gomes and Henriques, 2016). Therefore, finding effective non-antibiotic antimicrobials and alternative strategies to substitute the administration of antibiotics for mastitis treatment and control is vital. Painter et al., (2014) reported that the ability of *S. aureus* to cause a wide range of infections has been ascribed to its armory of various virulence factors, many of which are under the control of the quorum-sensing accessory gene regulator (**a*gr***) system of *S. aureus*. Singh and Ray (2014) demonstrated that *agr* plays a central role in staphylococcal pathogenesis. The *agr* system is composed of a two component signal transduction system that in response to a secreted auto-inducing peptide (**AIP**) controls virulence gene expression depending on cell density. At low cell density cell surface associated adhesion factors are produced, while at high cell density hemolysins and other secreted virulence factors are expressed (Le and Otto, 2015). Originally, the *agr* system was considered only to monitor the presence of *S. aureus* cell densities, but several studies have documented that other staphylococcal species produce AIP-like molecules, which inhibit *S. aureus agr* and toxin production (Otto et al., 2001; Canovas et al., 2016; Paharik et al., 2017). Therefore, knowledge of the microbial interactions between a variety of NAS species originating from dairy cows and dairy environment on the one hand, and *S. aureus* on the other hand, may ultimately lead to new ways of controlling infections with *S. aureus*. To the best of

our knowledge, there is no literature available that has investigated the crosstalk between *agr* quorum system of *S. aureus* and NAS isolated from milk as well as teat skin habitats of dairy cows at species level of NAS. The objectives of this study were to (1) investigate the distribution patterns of NAS species on quarter level from aseptic milk and teat skin samples in dairy herds with AMS, and (2) examine if the isolated NAS influences the expression of *S. aureus* virulence factors controlled by the *agr* quorum sensing system.

MATERIAL AND METHODS

Study population

Eight dairy herds with Danish Holstein cows were selected for participating in a project on *Streptococcus agalactiae* and *Staphylococcus aureus* IMI. The herds had to have AMS with ≥3 milking robots and Bulk tank milk (**BTM**) PCR cycle threshold (**Ct**) value ≤32 for *Streptococcus agalactiae.* About 30 to 40 lactating dairy cows were selected randomly from each herd based on the criteria of having no clinical mastitis, somatic cell count (**SCC**) ≥200,000 cells/mL at the previous milk recording, and not having been treated with antimicrobials during four weeks prior to sample collection. Teat skin swab and aseptic foremilk samples were collected from all quarters of selected cows. In the current study, samples from right hind and left front quarters of cows with an odd laboratory running number were included. Information about herd management practices and characteristics are listed in Table 1.

Sampling Procedures

Each herd was visited once to collect teat swab samples and aseptic quarter foremilk samples for bacterial culture. The farmers were asked to separate the selected cows and were fixed in head lockers or tied. Teat swab samples were collected according to the modified wet-dry method (Paduch et al., 2013). Briefly, the teat skin was sampled after cleaning with dry tissue paper. The first swab

(Dakla Pack) was moistened with ¼ Ringer's solution (Oxoid, Denmark) and rotated 360° around the teat about one cm from the teat canal orifice. The same procedure was carried out with the dry swab. Immediately after sampling, the tips of both swabs were transferred into one tube with 2 mL of sterile Ringer's solution.

Quarter milk samples were collected directly after harvesting the teat swab samples according to National Mastitis Council (1999) guidelines. Briefly, the teat end was thoroughly disinfected with cotton swabs drenched with ethanol (70%). Individual quarter foremilk samples were then aseptically collected in sterile screw-cap plastic tubes. New latex gloves were worn for each sampling procedure and each cow. Tubes containing the teat swabs and aseptic milk samples were stored in ice boxes and delivered to the microbiological laboratory within 12h. All study activities including farm visits, collection of samples and laboratory examination were carried out during the period from February to May 2017.

Laboratory Procedures

Bacterial culture and MALDI-TOF assay

Bacterial culture for milk samples was conducted in accordance with National Mastitis Council recommendations (1999). After vortexing, 0.01 mL of the milk sample from each quarter was streaked using disposable calibrated inoculating loops on a quarter of a calf blood agar and another 0.01 mL was streaked on a quarter of a chromogenic agar selective for Staphylococcus species (Sa*Select*, Bio-Rad, Marnes-la-Coquette, France), and incubated at 37°C for 48 h (Dolder et al., 2017). Bacterial culture of teat swab samples was performed according to the procedures of Paduch et al. (2013). Briefly, the teat swab samples were vortexed before removing the swab tips from the tubes. The agar plates were inoculated with 0.1 mL of the swab solution. The inoculum was spread with a sterile Drigalski spatula onto the whole calf blood agar and Sa*Select* agar for each quarter and were incubated at 37°C for 48 h.

Staphylococci species were identified on blood agar based on the phenotypic characteristics of their colonies including shape (round, glossy) according to according to National Mastitis Council guidelines (NMC, 2004) and their color on the selective media according to the manufacturer's instructions (Sa*Select*, Bio-Rad, Marnes-la-Coquette, France). We considered only quarter milk samples and teat skin swabs having three different *Staphylococcus* species per sample for further identification at species level. Cut-off ≥ 5 CFU on the plate was regarded as an acceptable cutoff point for definition of NAS IMI and NAS colonization of the teat apex (Thorberg et al., 2009).

All isolates of NAS species that were identified on bacterial culture were subcultured on calf blood agar and incubated for 24h at 37 ºC to be submitted freshly to MALDI-TOF (Microflex LT, Bruker Daltonics GmbH, Bremen, Germany) for identification. MALDI-TOF assay was conducted according to the manufacturer's instructions and Cameron et al. (2017) and all isolates were tested in triplicate. After two submissions to MALDI-TOF, the unidentified isolates were considered as "no possible identification". Cut-point threshold ≥ 1.7 was regarded as an acceptable and reliable threshold for identification of NAS species (Cameron et al., 2017). All identified NAS species isolates were stored in a sterile 10 % glycerol solution at −80°C for future use.

Qualitative Beta-Galactosidase Reporter Plate Assay

To examine if the NAS influence the expression of *S. aureus* virulence factors controlled by the *agr* quorum sensing system, a set of the identified NAS species was selected to represent all the identified species in milk and teat skin samples from the eight herds. NAS isolates were selected to represent all the identified NAS species (n=16) and to represent NAS isolates from milk and teat skin. *S. aureus* strain 8325-4 (Novick, 1967), which representing *agr* type I was used as a source of AIP-I containing supernatant. For the beta-galactosidase plate assays PC203 (*S. aureus* 8325-4, *spa::lacZ*), PC322 (*S. aureus* 8325-4, *hla::lacZ*), SH101F7 (*S. aureus* 8325-4, rnaIII::lacZ) (Chan and Foster, 1998; Canovas et al., 2016) were used. Strain 2898 of *Staphylococcus schleiferi* (positive

control) was used to produce a supernatant that inhibits *agr* activity (Canovas et al., 2016), while 8325-4 (AIP-I) supernatant was used to induce *agr*. The reporter assays and analysis of supernatants of NAS cultures were conducted as described by Nielsen et al. (2010) and Canovas et al. (2016). Briefly, bacteria (*S. aureus*) were grown on tryptone soy agar (TSA) plates containing erythromycin (5µg/mL), and the β-galactosidase substrate, 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) (150µg/mL). Overnight cultures were made by inoculating a single colony into 10 mL TSB in a glass vial, and let it shake vigorously (~ 200 rpm) at 37˚C overnight (16 h). Dilutions in NaCl were made from each overnight culture. About 1000 × dilution was used where the OD_{600} of the 10 × dilution adjusted to 0.35 before diluting then further to 0.0035. TSA was melted and cooled down in a water bath for approximately 45 minutes to 45˚C where X-gal (150µg/mL) and erythromycin (5µg/mL) were added. About 2 mL dilution of cells is mixed with 50 mL of media in Greiner plates. After the cells and the media were mixed well, the plates were let stand on the table to harden, dry in a LAF-bench for 45 min. In total 16 wells (14 test isolates plus one positive and one negative controls, Figure 1) were made manually with a sterile sharp iron drill to make a ring shaped cut through the agar. The little piece of agar in the middle of the ring was then removed using a sterile scalpel. About 20 µL cell-free supernatants of the test NAS strains were added to wells formed in TSA plates containing reporter strains carrying lacZ fusions to either one of the agr controlled virulence genes hla, spa, or rnaIII as well as the β-galactosidase substrate, and X-Gal. The plates were incubated at 37˚C for approximately 9 to 36 h until the blue color appeared on the plate. Downregulation was rated according to the presence or absence of the inhibition halo zone around the well. No zone means no effect, while presence of an inhibition halo zone means there is a downregulation effect and the degree of effect depend on the diameter of inhibition zone ranging from slight effect to severe effect.

To get the cell-free supernatants of the test strains, an overnight culture of the selected NAS strains was prepared and on the following day, about 2 mL of the cell culture in Eppendorf tube were spin

down in a table-top centrifuge at 8000 rpm for three min. We took 20 μL of the cell-free supernatants and placed them into the respective well.

Statistical analyses

To investigate if teat apex colonization with a specific NAS species increased the odds of IMI with the given species in the corresponding quarter, a logistic mixed regression model with herd and cow treated as random intercept was used. Different models were therefore performed for each of the NAS IMI species recovered from the quarter milk samples. Statistical analysis was carried out in R version 3.3.3 (The R Foundation for Statistical Computing). Results for all analyses were considered significant as those yielding a P-value ≤ 0.05.

RESULTS

NAS species in Milk and Teat skin and their association

Out of 150 cows considered in this study, eight cows were excluded for the reason of having dry quarters (n=16). In total, 80% (228/284) quarters from 142 cows harbored at least one NAS species. In total, MALDI-TOF identified 16 different NAS species. Out of these 16 species, 15 species were identified from teat skin, while only 10 species were identified from milk, and 9 species were identified from both sites, Table 2.

From milk, 105 isolates of NAS were identified from 94 quarters of 47 cows, while 268 isolates were identified on the teat skin of 190 quarters of 95 cows. The number of quarters with mixed (coinfections) infections (colonization or IMI) of NAS species (at least two different species) in teat skin swabs samples (37%, 70/190) was higher than the number of quarters with mixed infections in milk (12%, 11/94). *S. equorum* and *S. haemolyticus* were the most common combination of mixed NAS in teat skin (n=21 quarters) while in milk, no specific combination pattern but *S. epidermidis* was the most common partner (n= 6 quarters). Additionally, 18 isolates of *S. aureus* were identified

as coinfections with the different NAS species from milk (n=4) and teat skin (n=14) samples, Table 3.

The most prevalent NAS species identified from milk were *S. epidermidis* (50%, 52/105), *S. haemolyticus* (15%, 16/105), and *S. chromogenes* (11%, 11/105) accounting for 76% of all NAS isolates from milk. On the other hand, the most identified NAS species from teat skin were *S. equorum* (43%, 116/268), *S. haemolyticus* (15.7%, 42/268), and *S. cohnii* (14.2%, 38/268) accounting for 73% of all NAS isolates from teat skin. Remarkably, six NAS species including *S. capitis, S. sciuri, S. succinus, S. vitulans, S. saprophyticus,* and *S. piscifermentans* were not shown in milk, while *S. simulans* was the only NAS species that was not isolated from teat skin.

Distribution of NAS species varied among the eight herds (H1-H8) in both milk and teat skin samples. *S. equorum* was the most prevalent species in H1 (92%, 11/12), H2 (58%, 15/26), H3 (35%, 16/46), H4 (47%, 23/49), H5 (34%, 24/70) and H6 (29%, 21/72), while *S. haemolyticus* was most prevalent species in H7 (22%, 9/41) and *S. cohnii* in H8 (44%, 25/57). Teat apex colonization with *S. equorum* increased the odds of having IMI with *S. equorum* significantly, Table 2. Isolation of *S. chromogenes, S. cohnii, S. epidermidis, S. haemolyticus* and *S. xylosus* from teat skin was not found to increase odds of IMI for these NAS species.

Microbial Interactions of NAS species with S. aureus

Out of the total identified NAS isolates (n= 373), 81 isolates (32 milk and 49 teat skin), representing 16 different species from 58 dairy cows were selected to examine their ability to interfere with the *agr* quorum sensing system of *S. aureus*. In 58 (71.6%), 55 (67.9%), and 49 (60.5%) out of 81 of the staphylococcal supernatants of the tested NAS isolates, we observed reduced expressions of *hla,* rna-III, and *spa*, respectively (Figure 1; Table 3) indicating that NAS species interfere with the *agr* quorum sensing system of *S. aureus*.

NAS isolates of the same species from different herds showed different patterns on *agr* quorum sensing system of *S. aureus.* For example, isolates of *S. equorum* from milk of H1, H3 and H5 downregulated the *agr* quorum sensing system of *S. aureus*, while *S. equorum* isolates from H2, and H5 had no effect indicating the important role of herd characteristics and management on the pattern of microbial interactions.

The pattern of cross talk between NAS species and *S. aureus* virulence gene varied according to the involved NAS species. Our NAS isolates showed three main different patterns; (a) downregulation effect represented by *S. chromogenes* (milk), *S. simulans* (milk), *S. xylosus* (milk and teat), *S. saprophyticus* (teat), *S. warneri* (milk and teat), *S. haemolyticus* (milk and teat), *S. piscifermentans* (teat), and *S. arlettae* (teat), (b) no effect represented by *S. sciuri* (teat), and *S. vitulinus* (teat). The third pattern (c), variable effect, was represented by *S. epidermidis* (milk and teat), *S. equorum* (milk and teat), *S. hominis* (milk and teat), *S. cohnii* (milk and teat), and *S. succinus* (teat), Table 3.

DISCUSSION

To the best of our knowledge, this is the first study describing the distribution patterns of NAS species on quarter level from milk and teat skin in dairy herds with AMS. Furthermore, we have demonstrated for the first time the microbial interactions and cross-talk between different NAS species isolated from milk and teat skin, and *S. aureus* as mediated via the *agr* quorum sensing system and the resulting pattern on aspects of virulence and colonization of *S. aureus.*

NAS species from Milk and Teat skin and their association

We have identified 10 out of 16 NAS species in milk, where *S. epidermidis*, *S. haemolyticus* and *S. chromogenes* were most frequently isolated, confirming their major role in causing IMI in dairy herds with AMS. This comes in agreement with the findings of previous studies (Piessens et al., 2011; Dolder et al., 2017; Condas et al., 2017). However, other studies have reported different

predominant NAS species associated with bovine IMI (Supré et al., 2011; Fry et al., 2014). De Visscher et al., (2016a) concluded that *S. chromogenes, S. sciuri,* and *S. cohnii* were the predominant species causing IMI in freshly calved heifers and dairy cows. These variations among the findings of different studies could be caused by the differences in study design, type of milking system, species identification methods and criteria of IMI definition, different herd management, and species-specific characteristics of NAS (Zadoks and Watts, 2009).

Concerning the teat apex colonization, we identified 15 out of 16 identified NAS species with different frequencies. However; *S. equorum, S. haemolyticus*, and *S. cohnii* were identified as the most commonly isolated NAS species from teat skin across the eight herds. This finding indicates that teat skin is a natural habitat for a wider range of NAS species in comparison to those species found in milk, which could indicate that not all NAS species are adapted to the milk habitat or were equally able to invade the teat canal. In line with this statement, six NAS species including *S. capitis, S. sciuri, S. succinus, S. vitulinus, S. saprophyticus*, and *S. piscifermentans* have never been shown in milk, while *S. simulans* was the only NAS species to never have been isolated from teat skin. Falentin et al. (2016) reported that *Staphylococcus* was the dominant genus in the bovine teat microbiota (an average abundance of 23.8%) with *S. equorum* and *S. aureus* as the most commonly identified species (~13%) of staphylococci. Therefore, the wide range of NAS species could actually be part of the normal microbiota of the teat skin.

Our findings are comparable to the findings of previous studies, which isolated NAS from teat apex (Piessens et al., 2011; De Visscher et al., 2014, 2016b). Consistent with Braem et al. (2013) who found that *S. equorum,* and *S. haemolyticus* were the most prevalent NAS species on teat skin. Similarly, De Visscher et al. (2014) reported that the most prevalent species in the parlor-related extramammary niches were *S. cohnii, S. fleurettii,* and *S. equorum* in herd 1–3, respectively, while *S.*

haemolyticus and *S. sciuri* were present in all herds. Based on phenotyping, Taponen et al. (2008) found *S. equorum* and *S. sciuri*, and based on ribotyping, *S. succinus* and *S. xylosus*, as the predominant NAS species in extramammary samples (udder skin, teat apices and teat canals) of lactating dairy cows of one herd.

The distribution of NAS species differed widely across the eight herds. For instance, *S. arlettae* and *S. sciuri* were the most prevalent species in H7, while *S. haemolyticus* and *S. chromogenes* were the most prevalent species in H5. This marked variation in the distribution of NAS species across the study herds could indicate that the NAS distribution is "herd-specific". Similar findings were reported by Dolder et al. (2017) from Switzerland, and Condas et al. (2017) from Canada. Species-specific characteristics of NAS, herd-specific management and study design could be a possible explanation for the difference in species distribution between studies and herds. As shown in Table 1, our study herds showed different management practices in respect to type and management of robot, teat spray and robot disinfection, type of bedding and floor. Similar findings were reported in conventional dairy herds by De Visscher et al. (2014) who reported that *S. cohnii* was common on both teat apex and in milk, while *S. haemolyticus* in herd 1, *S. fleurettii* in herd 2 and *S. equorum* in herd 3 were more common on teat apex than in milk.

While teat apex colonization with *S. equorum* increased the odds of IMI in the same quarter, however, we could not find such significant association for other species such as *S. chromogenes, S. cohnii, S. epidermidis, S. haemolyticus,* and *S. xylosus*. A similar findings have been reported by previous studies (Piepers et al., 2011; Quirk et al., 2012; Braem et al., 2013). Quirk et al. (2012) found that *S. cohnii* was the only NAS that did not concurrently cause IMI and colonize the teat canal. Therefore, interchange between NAS species colonizing the teat skin and causing IMI is possible but that could be characteristic for specific NAS species (Adkins et al., 2018). De Visscher

et al. (2014) found a relationship between detection of NAS on teat apex and in milk, but could not determine the direction of the relationship. In other words: we do not know if NAS in milk originates from the teat skin or if the teat skin is colonized because of intramammary shedding of NAS. Dolder et al. (2017) suggested that the possible causes for a positive association might be a combination of distinct virulence factors, synergism in bacteria metabolism, and environmental conditions such as poor hygiene; however, the true underlying mechanisms remain unclear.

Microbial Interactions of NAS species with S. aureus

Previous studies have documented the *agr* cross-inhibition between *S. aureus* and other staphylococcal species mainly of human and non-bovine origin leading to an inhibition of the secreted virulence factors including major toxins such as alpha-hemolysin and the phenole soluble modulins (Otto et al., 2001; Canovas et al., 2016; Paharik et al., 2017). Our study confirms that similar patterns of microbial interactions exist between NAS species isolated from different habitats in dairy cows and *S. aureus*. Several staphylococcal species had the ability of cross interfering with the *S. aureus agr* quorum sensing system. These findings could be highly relevant to understand the role of NAS in udder health and may explain conflicting results reported from NAS in previous studies. Some studies reported that presence of NAS in the same habitat (e.g., milk) would provide a protective effect against IMI with *S. aureus* (De Vliegher et al., 2004; Piepers et al., 2011; Vanderhaeghen et al., 2014). Dos Santos Nascimento et al. (2005) reported that some NAS species from milk can produce antimicrobial substances “bacteriocins” inhibiting the growth of some major mastitis pathogens, including *S. aureus*. Recently, Goetz et al. (2017) reported that isolates of *S. chromogenes* and *S. simulans* significantly reduced biofilm formation in approximately 80% of the staphylococcal species tested, including *S. aureus*. Furthermore, previous studies confirmed the protective role of *S. chromogenes* against IMI with *S. aureus* (Matthews et al., 1990; De Vliegher et al., 2003, 2004).

375 Other research studies could not demonstrate a protective effect of NAS against major pathogens
376 including *S. aureus* (Vanderhaeghen et al., 2014) or *S. aureus* and *S. uberis* (Zadoks et al. 2001).
377 Previous reports showed that presence of NAS increased the odds of having a new *S. aureus* IMI
378 (Parker et al., 2007; Reyher et al., 2012). In the current study, we have shown different patterns for
379 different NAS species including NAS distribution within AMS herds, and sample type variation.
380 These different patterns could offer one or more explanation for the findings of the previous studies
381 on NAS epidemiology and characteristics. We want to highlight an important point of difference
382 between our findings and previous studies. Most of the previous studies investigated the relationship
383 and interaction between *S. aureus* and NAS species based specifically on the aspect of antimicrobial
384 interaction where NAS act by producing antimicrobial compounds that eliminate *S. aureus* from the
385 surrounding environment (De Vliegher et al., 2004; dos Santos Nascimento et al., 2005). Meanwhile,
386 our unique findings were based exclusively on investigation of the crosstalk between *S. aureus* and
387 NAS species via examining the influence of NAS species on the expression of *S. aureus* virulence
388 factors controlled by *agr* quorum sensing system.
389 For some species, all isolates (e.g., *S. chromogenes, S. xylosus, S. simulans* and *S. saprophyticus*)
390 repressed *agr* activity to some degree, whereas for other species (e.g., *S. epidermidis, S. equorum, S.*
391 *hominis*, and *S. cohnii*) only some of the isolates produced an *agr* inhibitory activity in culture
392 supernatants. Although, we do not know the exact mechanism of repression in these isolates, but we
393 anticipate that they produce AIP-like molecules that inhibit the *S. aureus* quorum sensing system.
394 Canovas et al., (2016) demonstrated that *S. schleiferi* produce an AIP variant that has very strong *agr*
395 repressing activity. However, in that study the staphylococcal species originated from different
396 animal host species such as dog, horse, cow, bird, and cat. Some NAS species are common to many
397 hosts such as *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. simulans*, and *S. xylosus*. While
398 other NAS species such as *S. caprae*, *S. chromogenes*, *S. felis*, *S. gallinarum* and *S. schleiferi* are the
399 most common species in small ruminants (Pengov, 2001), cattle (Carretto et al., 2005; Condas et al.,

2017), cats (Lilenbaum et al., 1999), chickens (Aarestrup et al., 2000), and dogs (Penna et al., 2000), respectively, while they are rare in other host species. Here, we show that NAS species found on teat skin and in milk of dairy cattle, also have the potential of repression *agr* activity. Our findings indicate that NAS species originating from the teat skin environment numerically appear to be more likely repressive to *S. aureus*, which has to be confirmed with a larger sample size and further investigation. It was reported that crosstalk involving agr interference has been observed as a result of co-habitual competition within the same ecological niche (Condas et al., 2017). In this study, we have identified 14 quarters harbor different NAS species having co-existence with *S. aureus* from milk (n=4) and from teat (n=14). Therefore, we speculate that the observed crosstalk may be explained partially by the co-habitual competition between *S. aureus* and NAS species within the same niche.

The selection criteria for the study herds and inclusion criteria of dairy cows and quarters should be taken in mind in terms of the generalizability of the obtained findings. We investigated NAS of cows with elevated SCC in AMS herds, which could differ from NAS derived from cows with low SCC or cows milked in other milking systems. In other words, AMS herds may have different NAS species with different characteristics compare to conventional milking systems. That could be argued by the no human contact to the udder tissue under AMS environment. Moreover, cows are milked several times (up to 5) daily with the same robot (Rasmussen, 2006). As the effect of NAS on SCC is species specific (Supré et al., 2011, Fry et al., 2014) with higher SCC reported from *S. chromogenes* and *S. simulans* (Fry et al. 2014), we may have selected for specific NAS species with more pronounced effect on SCC. The finding of interactions between *S. aureus* and different NAS species causing IMI and/or colonizing teat skin of dairy cows opens the door for identification of new and effective non-antibiotic anti-virulence strategies targeting *S. aureus* infections as alternative to antimicrobials or biocides used for *S. aureus* mastitis treatment and control. Further studies are necessary in the future

such as field studies with larger samples sizes and additional assays of NAS species isolates e.g. quantitative beta-galactosidase reporter plate assay to identify and quantify the cross-talk patterns between *S. aureus* and different NAS species.

CONCLUSION

In total, 15 different NAS species were identified from teat skin whereas 10 species were identified from milk. *S. epidermidis, S. haemolyticus* and *S. chromogenes* were the most prevalent species in milk accounting for 76%, while *S. equorum, S. haemolyticus* and *S. cohnii* were the most prevalent species in teat skin accounting for 73%. Staphylococcal supernatants of NAS species isolated from milk and teat skin interfered with the *agr* quorum sensing system of *S. aureus*. The pattern of cross talk between NAS species and *S. aureus* virulence gene expression varied according to the involved NAS species. Our NAS isolates showed three patterns; (a) downregulation effect e.g., *S. chromogenes* (milk), (b) no effect e.g., *S. sciuri* (teat), and (c) variable effect e.g., *S. epidermidis* (milk and teat). NAS species, habitat type, and herd factors affect NAS and *S. aureus* crosstalk patterns. The findings of this study will boost our knowledge and understanding of the epidemiology of NAS species and their relation with *S. aureus* IMI and/or colonization of teat skin of dairy cows.

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593

Table 1. Description of herd management practices in the study herds with automatic milking systems with respect to housing, milking and robot hygiene.

Herd code	Herd size [a]	Milk production energy corrected milk/cow/year	Type of robot (no)	Robot cleaning (per day)	Robot disinfection	Teat spray	Floor	Bedding [b]	Additions to beds	No of cows with SCC > 200,000 cells/mL	No of cows sampled
H1	267	10,973	Lely A4 (4)	2 x robot automatic wash 2 x high pressure washer Brushes in chlorine	Astri-L[c]	JOPO winterspray (0.3 % iodine)	Solid, slatted in front of robots and water trough (1/3)	Chopped straw 2 x day	Hydrated lime	43	13
H2	198	11,098	Lely A2 (3)	2 x robot automatic wash 2 x high pressure washer	Astri-L[c]	Kenostart (0.3 % iodine)	Solid, slatted in front of robots and water trough (1/3)	Chopped straw 3 x week	Ökosan GFR[g]	43	17
H3	344	10,733	Lely A2 (7)	2 x robot automatic wash 2 x high pressure washer Brushes in washing machine	Astri-L[c]	HM VIR GOLD (1 % lactic acid)	Solid, slatted in front of robots and water trough (1/3)	Chopped straw 2 x day	Sanibed[h]	74	20
H4	298	11,412	Lely A3 (5)	3 x robot automatic wash 2 x soap + brush + water Brushes in chlorine	Oxivit Aktiv Plus[d]	NOVA DIP (0.75 % iodine)	Statted, rubber in front of robots and feeding table	Chopped straw 1 x day	Limestone	60	20
H5	218	9,024	Lely A2 (4)	2 x robot automatic wash 2 x foam + water Brushes in acid	Oxivit Aktiv Plus[d]	JOPO winterspray (0.3 % iodine)	Slatted	Chopped straw 1 x day	Hydrated lime	49	20
H6	247	11,701	Lely A3 (4)	3 x robot automatic wash 2 x high pressure washer	Solox[e]	Kenostart (0.3 % iodine)	Slatted	Chopped straw 2 x day	Basic Strømiddel Destek[m]	59	20
H7	333	11,909	DeLaval (6)	2 x robot automatic wash 1-2 x high pressure washer DeLaval soap + water	PeraDis[f]	ProActive Plus (0.15 % iodine)	Statted, rubber in front of robots and feeding table	Chopped straw 2 x day	Limestone	50	20
H8	244	11,020	DeLaval (4)	2 x robot automatic wash 1-2 x high pressure washer	PeraDis[f]	ProActive Plus (0.15 % iodine)	Slatted	Chopped straw + wood showings 1 x day	Destek CombiRen[n]	79	20

[a] Includes both lactating and dry cows; [b] all herds had stalls with mattresses; [c] pH < 3, hydrogenperoxide, peracetic acid, and acetic acid; [d] pH = 1, peracetic acid, acetic acid, and hydrogenperoxide; [e] pH < 1, peracetic acid, hydrogenperoxide, and acetic acid; [f] pH = 0.5, hydrogenperoxide, and peracetic acid; [g] pH = 12, calcium compounds; [h] pH = 2.9, Salicylic acid; [m] pH = 8, Tosylchloramide sodium; [n] pH = 8-10, Tosylchloramide sodium

599 Table 2. Species distribution and association of NAS isolates from aseptic quarter milk and teat skin

600 samples collected from 142 cows (284 quarters) in eight dairy herds with automatic milking systems.

NAS species (n) [a]	Sample type (%)		OR [b] (95% CI)	P-value
	Milk (n=105)	Teat (n=268)		
Staphylococcus arlettae (12)	1 (0.9)	11 (4.1)	---	---
Staphylococcus capitis (3)	---	3 (1.1)	---	---
Staphylococcus chromogenes (16)	11 (10.5)	5 (1.9)	7.6e-1 (NA - 2.4e+7)	0.85
Staphylococcus cohnii (43)	5 (4.8)	38 (14.2)	2.23 (0.11 - 15.6)	0.48
Staphylococcus epidermidis (60)	52 (49.5)	8 (3.0)	0.88 (0.05 - 5.07)	0.90
Staphylococcus equorum (122)	6 (5.7)	116 (43.3)	4.9e-1 (NA - 8.9e+7)	0.016*
Staphylococcus haemolyticus (58)	16 (15.2)	42 (15.7)	1.13 (0.17 - 4.24)	0.55
Staphylococcus hominis (17)	3 (2.9)	14 (5.2)	---	---
Staphylococcus piscifermentans (2)	---	2 (0.8)	---	---
Staphylococcus saprophyticus (5)	---	5 (1.9)	---	---
Staphylococcus sciuri (9)	---	9 (3.4)	---	---
Staphylococcus simulans (2)	2 (1.9)	---	---	---
Staphylococcus succinus (2)	---	2 (0.8)	---	---
Staphylococcus vitulinus (1)	---	1 (0.4)	---	---
Staphylococcus warneri (2)	1 (0.9)	1 (0.4)	---	---
Staphylococcus xylosus (19)	8 (7.6)	11 (4.1)	3.8e-1 (NA - 3.4e+7)	0.49

601 [a] *Staphylococcus* arlettae, *S. warneri*, and *S. hominis* were not considered in the statistical analysis
602 because of the few number of observations (< 5), while *S. capitis*, *S. piscifermentans*, *S.*
603 *saprophyticus*, S. *sciuri*, *S. simulans*, S. *succinus*, and *S. vitulinus* were not isolated from milk and/or
604 teat skin.
605 [b] OR= Odds ratio; * significance at < 0.05

606 Table 3: Results of 81 staphylococcal strains, their origin, and hla, spa, and RNA III -regulation activity with regard to sample type

NAS species	hla				spa				rna_III				Overall number of NAS per sample type		Number of *S. aureus* isolated from same sample type	
	Milk (n)		Teat (n)		Milk (n)		Teat (n)		Milk (n)		Teat (n)		Milk (n)	Teat (n)	Milk (n)	Teat (n)
	Yes*	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No				
Staphylococcus arlettae (n=5)	-	1	4	-	-	1	4	-	-	1	4	-	1	4	-	3
Staphylococcus capitis (n=3)	-	-	2	1	-	-	1	2	-	-	2	1	-	3	-	-
Staphylococcus chromogenes (n=8)	4	-	4	-	4	-	4	-	4	-	4	-	4	4	-	-
Staphylococcus cohnii (n= 7)	2	1	1	3	2	1	1	3	2	1	1	3	3	4	1	4
Staphylococcus epidermidis (n= 10)	2	3	4	1	1	4	2	3	1	4	2	3	5	5	1	1
Staphylococcus equorum (n= 9)	3	2	3	1	3	2	3	1	3	2	3	1	5	4	1	3
Staphylococcus haemolyticus (n= 8)	4	-	4	-	1	3	3	1	4	-	4	-	4	4	-	-
Staphylococcus hominis (n= 6)	1	2	1	2	1	2	-	3	1	2	1	2	3	3	-	-
Staphylococcus piscifermentans (n= 2)	-	-	2	-	-	-	2	-	-	-	2	-	-	2	-	1
Staphylococcus saprophyticus (n= 4)	-	-	4	-	-	-	3	1	-	-	4	-	-	4	-	1
Staphylococcus sciuri (n= 4)	-	-	-	4	-	-	-	4	-	-	-	4	-	4	1	-
Staphylococcus simulans (n= 2)	2	-	-	-	2	-	-	-	2	-	-	-	2	-	-	-
Staphylococcus succinus (n= 2)	-	-	1	1	-	-	1	1	-	-	1	1	-	2	-	-
Staphylococcus vitulinus (n= 1)	-	-	-	1	-	-	-	1	-	-	-	1	-	1	-	-
Staphylococcus warneri (n= 2)	1	-	1	-	1	-	1	-	1	-	1	-	1	1	-	-
Staphylococcus xylosus (n= 8)	4	-	4	-	4	-	4	-	4	-	4	-	4	4	-	1
Total (N=81)	23	9	35	14	20	12	29	20	22	10	33	16	32	49	4	14

607 * *Downregulation was rated according to the presence or absence of the inhibition halo zone around the well where: No zone; means no effect,*
608 *while Yes (presence of zone); means there is a downregulation effect and that effect ranged varied according to the diameter of inhibition zone*
609 *from slight effect to severe effect*

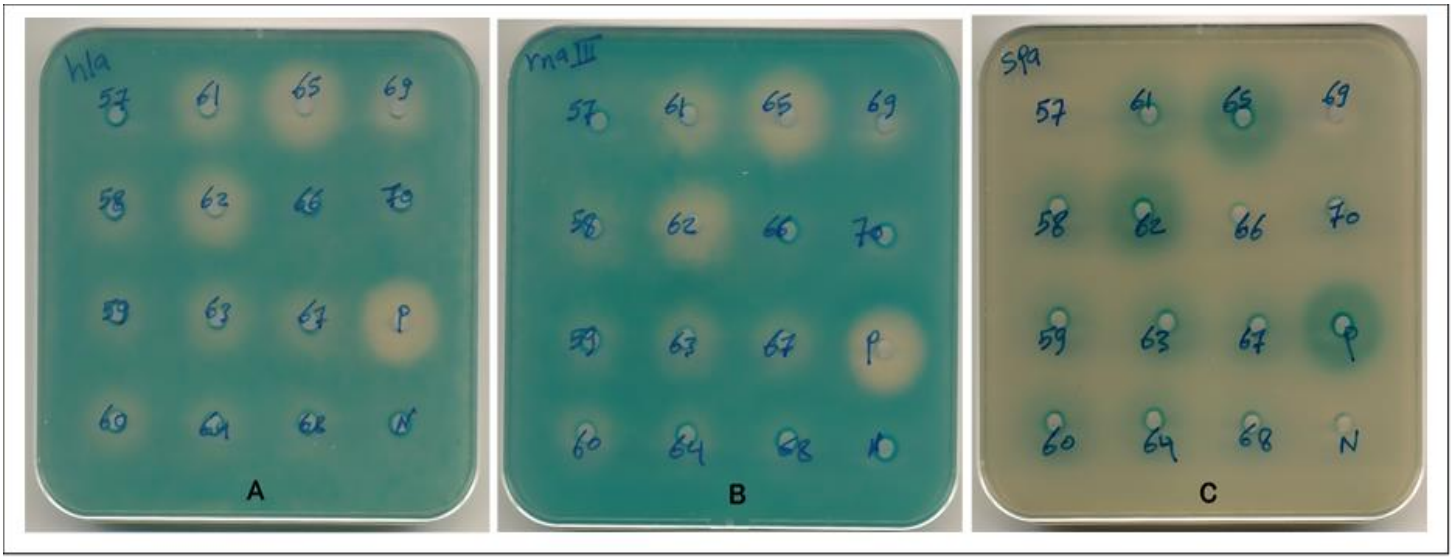

Figure 1. Modulation of *Staphylococcus aureus* virulence gene expression by non-aureus staphylococcal culture supernatants. TSA agar plates (with erythromycin and X-gal) containing **(A)** the *hla-lacZ* (PC322; Eryr), **(B)** the *rnaIII-lacZ* (SH101F7; Ery^r), or **(C)** the *spa-lacZ* (PC203; Ery^r) reporter strain of *Staphylococcus aureus* were exposed to 20 mL (in pre-drilled wells) of supernatants from centrifugation (8000 rpm for 60s) of overnight cultures of strains 57(*Staphylococcus equorum*), 58(*Staphylococcus epidermidis*), 59(*Staphylococcus piscifermentans*), 60(*Staphylococcus xylosus*), 61(*Staphylococcus chromogenes*), 62(*Staphylococcus arlettae*), 63(*Staphylococcus haemolyticus*), 64(*Staphylococcus piscifermentans*), 65(*Staphylococcus arlettae*), 66(*Staphylococcus sciuri*), 67(*Staphylococcus haemolyticus*), 68(*Staphylococcus xylosus*), 69(*Staphylococcus haemolyticus*), and 70(*Staphylococcus cohnii*). P (positive control): Strain 2898 of *Staphylococcus schleiferi*. N (negative control): NaCl. Zones appeared between 9 and 36h of incubation at 37 °C.
This figure is representative of one set of screening plates.

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