Distribution of non-*aureus* staphylococci from quarter milk, teat apices, and rectal feces of dairy cows, and their virulence potential

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ABSTRACT

Non-*aureus* staphylococci (NAS) are predominantly isolated from bovine milk samples of quarters suffering from subclinical mastitis. They are also abundantly present on dairy cows' teat apices and can be recovered from bovine fecal samples, as recently described. Differences in ecology, epidemiology, effect on udder health, and virulence or protective traits have been reported among the species within this group. The objectives of this study were (1) to describe the species-specific distribution of NAS in 3 bovine-associated habitats, namely quarter milk, teat apices, and rectal feces, and (2) to evaluate the virulence potential of NAS by comparing their distribution in contrasting milk sample strata and the presence of selected virulence genes. A cross-sectional, systematic sampling procedure was followed in 8 dairy herds that participated in the local Dairy Herd Improvement program in Flanders, Belgium. Quarter milk samples ($n = 573$) were collected from 144 lactating cows in 8 herds. In 5 of the 8 herds, teat apex swabs ($n = 192$) were taken from 15 lactating cows, before and after milking, and from 18 dry cows. In the same 5 herds, rectal feces were sampled from 80 lactating cows ($n = 80$), taking into account that a cow could only serve as the source of one type of sample. In addition, milk samples of all clinical mastitis cases were continuously collected during the 1-yr study period from March 2017 to March 2018 in the 8 herds. In total, 1,676 *Staphylococcus* isolates were phenotypically identified and subjected to MALDI-TOF mass spectrometry. Thirty-three, 98, and 28% of all quarter milk, teat apex, and rectal fecal samples were NAS-positive, respectively, reaffirming the presence of NAS in rectal feces. The overall predominant species in the 3 habitats combined were *Staphylococcus haemolyticus*,

Staphylococcus chromogenes, and *Staphylococcus hominis*. Four, 16, and 12% of the healthy quarters (quarter milk somatic cell count $\leq 50,000$ cells/mL of milk), quarters with subclinical mastitis (quarter milk somatic cell count $> 50,000$ cells/mL of milk), and quarters with clinical mastitis, respectively, were NAS-positive, suggesting that the potential to cause (mild) clinical mastitis is present among NAS. This was substantiated by comparing the presence of virulence genes of NAS isolates originating from contrasting milk sample strata (healthy quarters and quarters with clinical mastitis).

Key words: mastitis, non-*aureus* staphylococci, rectal feces, teat apex

INTRODUCTION

Non-*aureus* staphylococci are the most frequently isolated bacteria causing IMI in dairy cows worldwide (Sampimon et al., 2009b; Schukken et al., 2009; Condas et al., 2017b). They also colonize teat apices of both lactating and dry cows (Braem et al., 2013; De Visscher et al., 2016b; Adkins et al., 2018a) and can be present in feces of dairy cows (Wuytack et al., 2019). Whether milk, teat apices, and feces have comparable NAS distributions remains unclear.

The relevance of NAS IMI for bovine udder health and milk yield is still not entirely clear. Non-*aureus* staphylococci, as a group, are minor pathogens, but species- and strain-specific research revealed variation in virulence among NAS species (Vanderhaeghen et al., 2014). An increase in SCC comparable to a *Staphylococcus aureus* IMI (Supré et al., 2011; Fry et al., 2014; Valckenier et al., 2020) and the ability to cause persistent IMI (Rajala-Schultz et al., 2009; Mørk et al., 2012; Valckenier et al., 2020) or even mild clinical mastitis (Taponen et al., 2006; Simojoki et al., 2009; Verbeke et al., 2014) have been reported. At the same time, protective effects against other major mastitis pathogens of NAS IMI or NAS teat apex colonization have been described (De Vliegher et al., 2004; Piepers et al., 2009)

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and several authors describe little to no negative effects on udder health (Matthews et al., 1991; Reyher et al., 2012) and milk yield (Pearson et al., 2013; Piepers et al., 2013; Valckenier et al., 2019). Additional research into the virulence potential of NAS (e.g., by comparing their presence in milk samples from healthy quarters and from quarters with clinical mastitis and by comparing the frequency of virulence genes in NAS isolates originating from these 2 opposite strata) would be helpful in that respect. Methicillin-resistance and virulence determinants may be combined on the staphylococcal chromosomal cassette *mec* (**SCC*mec***) element without reducing the competitive fitness of the *Staphylococcus* spp. (Diep et al., 2008; Queck et al., 2009; Pozzi et al., 2012). The presence of methicillin-resistance genes was, therefore, determined as well.

The main objective of this study was to broaden our knowledge of the species-specific NAS presence and distribution in 3 bovine-associated habitats, namely quarter milk, teat apices, and rectal feces. Second, the (species-specific) virulence potential of NAS was evaluated by comparing their presence in milk samples originating from quarters with a low or elevated quarter milk SCC or with signs of clinical mastitis, and by determining the presence of selected virulence and methicillin-resistance genes in NAS originating from contrasting milk sample strata (low quarter milk SCC and clinical mastitis).

MATERIALS AND METHODS

Herds, Cows, and Samples

Eight commercial dairy herds (**H1–H8**) were enrolled in this observational study, conducted between March 2017 and March 2018. All herds participated in the local DHI program in Flanders, Belgium, with an interval of 4 to 6 wk between 2 test-days (CRV, Arnhem, the Netherlands). The herds had an average to good udder health management based on the implementation of the mastitis prevention and control program of the National Mastitis Council (**NMC**; National Mastitis Council, 2010). During the study, the average number of lactating cows per herd was 118 dairy cows (ranging from 50 to 237 cows) with a yearly average milk production per cow of 9,304 kg of milk (ranging from 8,646 to 12,059 kg of milk) and a geometric mean of the monthly bulk milk SCC of 121,000 cells/mL of milk (ranging from 77,000 to 188,000 cells/mL of milk). The teat disinfection products used before milking were either none (H2, H3, H5, and H7) or foam based on low concentration of lactic acid (H1, H4, H6, and H8; Keno pure, CID LINES, Ieper, Belgium, <https://www.cidlines.com/en-INT/kenotmpure>) and after the milk-

ing process were either based on iodine (H1, H2, H4, H6, and H8; IoShield, Ecolab, St. Paul, MN, <https://en-uk.ecolab.com/offerings/post-milking-udder-care/io-shield>; VelouCid, Ecolab, <https://en-uk.ecolab.com/offerings/post-milking-udder-care/veloucid-d>; Blockade, DeLaval, Cardiff, UK, <https://www.delaval.com/en-gb/our-solutions/milking/udder-health--hygiene/teat-dips/>) or chloordioxide (H3, H5, and H7; Dippinggold Comfort, Heemskerk, Diessen, the Netherlands, <https://dailymed.nlm.nih.gov/dailymed/fda/fdaDrugXsl.cfm?setid=f93303bd-451c-4fd3-99d7-370e2eb444e9&type=display>), following the manufacturers' guidelines.

A single cross-sectional sampling of each herd was performed during which quarter milk samples from approximately 25% (maximum 80 milk samples per herd, Table 1, Figure 1) of the lactating animals per herd were collected via systematic random sampling for bacteriological culturing based on the guidelines of the NMC (Hogan et al., 1999). As well, milk samples of all quarters with clinical signs of mastitis were collected secundum artem by the producers during the entire study period. Based on the severity of the disease signs, clinical mastitis cases were categorized as mild, moderate, or severe, following the scoring system described by Kalmus et al. (2013). The quarter milk SCC (**qSCC**) was determined from all milk samples except from quarters with signs of clinical mastitis, using a Direct Cell Counter (DeLaval, Tumba, Sweden) and used to stratify the nonclinical samples in low qSCC ($\leq 50,000$ cells/mL) milk samples (~healthy quarters) and elevated qSCC ($> 50,000$ cells/mL) milk samples (considered as quarters with subclinical mastitis; Leitner et al., 2003; Barbano et al., 2006; Potter et al., 2018).

In 5 of the 8 herds (H1, H2, H3, H4, and H5), swabs from teat apices (Table 1, Figure 1) were collected at the same time the milk samples were collected. The 4 teat apices of both lactating and dry cows (total number of animals equaled approximately 10% of the total number of lactating cows per herd) were sampled in a systematic random way [excluding cows already sampled for milk or feces (see further)]. Approximately one-third of the sampled teat apices originated from lactating cows and two-thirds from dry cows. If the number of dry cows was insufficient at the moment of the cross-sectional sampling, extra teat apices of lactating cows were sampled. The teat apices of the lactating cows were sampled twice, once before and once after milking at sampling day. Samples were collected with a dry cotton swab (Copan, Novolab, Belgium) as described by De Visscher et al. (2014). The teat disinfection products used before milking are described above.

In the same 5 herds, rectal feces samples (Table 1, Figure 1) were collected from approximately 25% of the

lactating cows per herd (excluding cows sampled for milk or teat apices) via systematic random sampling, using double gloves reflecting a guarded sampling technique (Wuytack et al., 2019).

All samples were transported under cooled conditions (4°C) to the Mastitis and Milk Quality Research Laboratory (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium).

Bacterial Isolation and Identification

Quarter milk samples were plated with a 0.01-mL loop on esculin blood agar (Oxoid, Aalst, Belgium), MacConkey agar (Oxoid), and mannitol salt agar (MSA, Chapman medium, Oxoid), a semi-selective agar for staphylococci (De Visscher et al., 2013). Swabs collected from the teat apices were streaked on MSA to prevent overgrowth of other bacteria, which is frequently observed in samples from teat apices (Lugosmya et al., 2012; De Visscher et al., 2013). As described by Wuytack et al. (2019), rectal feces (1 g) were first diluted in 9 mL of 0.9% physiological salt solution (9 g of NaCl/L, Eurovet, Bladel, the Netherlands) and then plated with a 0.01-mL loop on MSA. All plates were incubated at 37°C and examined after 24 and 48 h. All phenotypically different colony types (with ≥ 1 colony) per plate were counted and subcultured on blood agar (Columbia agar with 5% sheep blood, Oxoid) and phenotypical bacteriological identification was performed following the NMC procedure guidelines (Hogan et al., 1999). The isolates identified as *Staphylococcus* spp. were stored at -80°C until further analysis.

All isolates phenotypically identified as *Staphylococcus* spp. were submitted to MALDI-TOF with a custom reference-spectra expanded database for bovine-

associated NAS (Cameron et al., 2018). The MALDI-TOF assay was performed using the direct transfer method with a cut-off value of ≥ 1.700 for NAS isolates (Cameron et al., 2017). In addition, the MALDI library was updated as described by Cameron et al. (2017) with the following missing species: *Staphylococcus jet-tensis*, *Staphylococcus lentus*, *Staphylococcus rostri*, and *Staphylococcus saprophyticus* and some additional bovine isolates belonging to *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus fleuretti*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus*, and *Staphylococcus simulans*.

Descriptive Analysis

To picture the overall distribution of NAS in the different habitats, all isolates originating from quarter milk (with or without an elevated qSCC or clinical signs), teat apices, and rectal feces were included for the descriptive analysis. Quarter milk samples harboring more than 2 different microorganisms on either MSA, esculin blood agar, and MacConkey agar were considered contaminated and excluded from the analysis. For this analysis, NAS were considered as one group, meaning that samples could harbor isolates belonging to more than 2 different NAS species.

The (species-specific) virulence potential of NAS was evaluated based on their distribution among the contrasting milk sample strata (healthy quarters with a qSCC $\leq 50,000$ cells/mL and quarters with clinical signs) and by comparing the presence of 15 virulence genes in the NAS isolates ($n = 60$; 17 from quarters with a low qSCC and 43 from clinical quarters). To do so, NAS isolates from quarter milk samples harboring NAS only were used, meaning that quarter milk sam-

Table 1. The number of samples collected per dairy herd and per habitat (quarter milk, teat apices, and rectal feces) during the cross-sectional and continuous sampling period of 1 yr for clinical mastitis cases

Habitat	Herd (H)								Total
	H1 (44) ¹	H2 (94)	H3 (62)	H4 (64)	H5 (67)	H6 ² (187)	H7 ² (203)	H8 ² (93)	
	Number of samples								
Quarter milk	59	115	64	98	100	116	159	92	803
Healthy quarters (geometric mean 20,000 cells/mL)	16	37	37	23	52	41	47	59	312
Subclinical signs (geometric mean 152,000 cells/mL)	32	42	27	51	16	39	33	21	261
Clinical signs	11	36	—	24	32	36	79	12	230
Teat apices	32	44	40	32	44	— ²	—	—	192
Lactating cows	16	24	32	16	32	—	—	—	120
Before milking	8	12	16	8	16	—	—	—	60
After milking	8	12	16	8	16	—	—	—	60
Dry cows	16	20	8	16	12	—	—	—	72
Rectal feces	11	20	16	16	17	—	—	—	80

¹The number of lactating cows present per herd during the cross-sectional sampling is shown in parentheses.

²In herds 6, 7, and 8, only quarter milk samples were collected.

Figure 1. Overview of the study design. qSCC = quarter milk somatic cell count; max. = maximum.

ples harboring bacteria other than NAS as evidenced on MSA, esculin blood agar, or MacConkey agar (or a combination of these) were excluded for this analysis.

Statistical Analysis

Using ANOVA, differences between NAS as a group and major pathogens in effect on qSCC were analyzed statistically as well as the effect of the most prevalent NAS species (*S. chromogenes* and *S. haemolyticus*; IBM SPSS Statistics version 25, Armonk, NY). Only milk samples harboring microorganisms in pure culture were included for this analysis. A natural log-transformation of qSCC was performed to normalize the data. Bonferroni-adjusted *P*-values were generated to adjust for multiple comparisons.

PCR Assay

The DNA was extracted from the 60 NAS isolates originating from the 2 contrasting milk sample strata with the use of the commercial DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) based on the

manufacturer's instructions. One isolate originating from a healthy quarter from herd 5 was, however, not revivable from the -80°C .

Kit reagents without sample were used as negative control. The virulence and methicillin-resistance associated genes tested with the use of specific PCR amplifications were *agrA* (accessory gene regulator protein), *bap* (biofilm-associated protein), *cap5H* and *8H* (CP5 and CP8 synthesis enzyme), *clfA* (clumping factor), *coa* (coagulase), *eno* (laminin binding protein), *fnbpA* and *B* (fibronectin-binding protein A and B), *hla* (α -hemolysin), *hly* (β -hemolysin), *ica* (polysaccharide intercellular adhesion), *mecA* and *mecC* (methicillin resistance), *nuc* (thermonuclease), *ssl7* (staphylococcal superantigen-like protein), and *vwb* (von-Willebrand factor binding protein). The PCR reactions for the virulence and methicillin-resistance genes were performed according to Wang et al. (2016) with the following modification: the PCR reaction mixture (25 μL) contained 2X ALLin HS Red Taq Mastermix (Labconsult, Brussels, Belgium) instead of 2X Taq PCR MasterMix. The primer sequences, annealing temperatures, and positive controls used for the detection of the virulence

and methicillin-resistance genes are shown in Table 2. In each PCR run, a positive and negative control (without DNA template) were included. The PCR products were analyzed by agarose gel electrophoresis (1.5% wt/vol), previously stained with ethidium bromide (Sigma-Aldrich, Overijse, Belgium).

RESULTS

Overall Distribution of NAS

Quarter Milk, Teat Apices, and Rectal Feces.

In total, 1,676 isolates were phenotypically identified as *Staphylococcus* spp. and submitted to the MALDI-TOF MS. In sum, 166 isolates had either no identification (51 isolates, score <1.700) or were identified as *Aerococcus* spp., *Arthrobacter* spp., *Corynebacterium* spp., *Enterococcus* spp., *Kocuria* spp., or *Micrococcus* spp. (score ≥1.700), resulting in 1,510 *Staphylococcus* isolates (score ≥1.700). The NAS distribution in the 3 examined habitats, quarter milk, teat apices, and rectal feces, is tabulated in Table 3. For 9 species, isolates were present in all 3 habitats in the same herd, namely *Staphylococcus arlettae*, *Staphylococcus auricularis*, *S. chromogenes*, *Staphylococcus cohnii*, *Staphylococcus devriesei*, *Staphylococcus equorum*, *S. haemolyticus*, *Staphylococcus hominis*, and *Staphylococcus vitulinus*. *Staphylococcus haemolyticus* and *S. hominis* were even represented in all habitats in 2 (herds 1 and 5), and 4 (herds 1, 2, 3, and 4) herds, respectively. Other species were found in only 1 habitat, namely *Staphylococcus agnetis*, *Staphylococcus kloosii*, and *Staphylococcus pettenkoferi* on teat apices and *Staphylococcus hyicus*, *Staphylococcus schleiferi*, and *Staphylococcus warneri* in quarter milk.

Quarter Milk. In total, 803 quarter milk samples originating from 749 quarters of 301 cows, were collected, of which 39%, 32%, and 29% were originating from healthy quarters, quarters with subclinical mastitis, and quarters with signs of clinical mastitis, respectively (Table 1). Table 4 gives an overview of the NAS-positive samples per herd for the overall distribution of NAS. Allowing for pathogens other than NAS to be present, 33% of the quarter milk samples were NAS-positive (range 18 to 50% per herd), harboring 22 NAS species (range 5 to 15 species per herd). The 5 most prevalent species were *S. chromogenes* (10% positive quarter milk samples), *S. haemolyticus* (9%), *S. equorum* (7%), *S. hominis* (5%), and *S. cohnii* (3%). *Staphylococcus chromogenes*, *S. haemolyticus*, and *S. hominis* were present in quarter milk on all farms.

Teat Apices. In total, 192 teat apex samples from 33 cows in 5 herds were sampled (Table 1). This included samples from 15 lactating cows sampled before

and after the milking process, as well as samples from 18 dry cows. All teat apex samples harbored NAS species except for 8 (4.2%), which were all taken after the milking process. Up to 8 different NAS species per teat apex sample could be identified, resulting in 830 NAS isolates, belonging to 22 different NAS species (Table 4). The 5 most frequently isolated NAS species were *S. haemolyticus* (70% positive teat apex samples), *S. equorum* (33%), *S. chromogenes* (23%), *S. cohnii* (19%), and *S. devriesei* (15%; Table 3). *Staphylococcus cohnii* was present in only 2 herds, whereas *S. devriesei* was present in 4 of the 5 sampled herds. *Staphylococcus auricularis*, *S. chromogenes*, *S. equorum*, and *S. haemolyticus* were isolated from teat apices on every farm.

Table 5 outlines the distribution of NAS species from the teat apices of the lactating cows (n = 15), swabbed before and after milking, and dry cows for the different herds. *Staphylococcus haemolyticus* was found on all sampled teat apices before milking in herd 1. *Staphylococcus chromogenes* and *S. haemolyticus* were present on all sampled teat apices from dry cows in herd 3. *Staphylococcus kloosii*, *S. lentus*, and *S. saprophyticus* were only picked up from teat apices of the lactating cows before the milking process, whereas *S. agnetis* and *S. epidermidis* were only found after milking. The following species could only be isolated from the teat apices of dry cows: *S. fleurettii*, *S. pettenkoferi*, *S. rostri*, and *S. simulans*. *Staphylococcus agnetis*, *S. kloosii*, *S. lentus*, and *S. saprophyticus* could not be retrieved from teat apices of dry cows.

Rectal Feces. Rectal fecal samples of 80 cows from 5 herds were collected (Table 1). Twenty-eight percent (ranging from 6% to 55% per herd) was NAS-positive, resulting in 33 different isolates belonging to 12 species (Table 4). Table 3 describes the distribution of NAS species for the different herds. All herds had at least one dairy cow that was shedding NAS. The most prevalent species were *S. hominis* (31% of the rectal fecal samples), *S. epidermidis* (15%), *S. cohnii* (12%), and *S. haemolyticus* (8%). Other NAS species present in rectal feces were *S. arlettae*, *S. auricularis*, *S. capitis*, *S. chromogenes*, *S. devriesei*, *S. equorum*, *S. lentus*, and *S. vitulinus*.

Virulence Potential of NAS in Contrasting Milk Strata

NAS Presence. During the cross-sectional sampling, 573 clinically healthy quarters belonging to 144 cows were sampled (Table 1). Three-quarters were blind. Three hundred twelve (54%) and 261 (46%) quarter milk samples had a qSCC ≤ or >50,000 cells/mL of milk, respectively. During the sampling period of 1 yr, 188 cows showed signs of clinical mastitis. Of those 188 cows, 28 cows had more than one clinical mastitis case

Table 2. Target genes, primers, and PCR conditions used for the amplification of virulence and methicillin-resistance genes

Target gene	Protein	Primer pair ¹	Primer sequence (5'-3')	Temperature (°C)	Positive control	Reference
<i>mecA</i>	Antimicrobial susceptibility	<i>mecA1</i>	AAA ATC GAT GGT AAA GGT TGG C	55	<i>Staphylococcus aureus</i> ATCC 33591	Murakami et al. (1991)
<i>mecC</i>	Methicillin resistance	<i>mecA2</i>	AGT TCT GCA GTA CCG GAT TTG C	55	<i>S. aureus</i> LGA 251	Garcia-Álvarez et al. (2011)
<i>coa</i>	Exoenzymes	<i>mecC</i>	TCA CCA GGT TCA AC(Y) CAA AA	55		
	Coagulase	<i>mecC</i>	CCT GAA TC(W) GCT AAT AAT ATT TC	55		
<i>vwb</i>	von Willebrand factor binding protein		ATA GAG ATG CTG GTA CAG G	55	<i>S. aureus</i> ATCC 29213	Mahmoudi et al. (2017)
			GCT TCC GAT TGT TCG ATG C	55	<i>S. aureus</i> ATCC 25923	Bjerketorp et al. (2002)
<i>nuc</i>	Thermonuclease	<i>nuc-F</i>	AAT ATA CCA TGG TGG TTT CTG GGG AGA	57	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
		<i>nuc-R</i>	AGA AT			
<i>hla</i>	Exotoxins	<i>hla-F</i>	TTT GCC ATT ATA TAC TTT ATT GAT	57		
	α-Hemolysin precursor	<i>hla-R</i>	GCG ATT GAT GGT GAT ACG GGT	57	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
<i>hlb</i>	β-Hemolysin precursor	<i>hla-R</i>	AGC CAA GGC TTG ACG AAC TAA AGC	58	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
<i>ssl7</i>	Staphylococcal superantigen-like protein	<i>hla-R</i>	GGT TTA TAC ATC CCA TGG C	58	<i>S. aureus</i> ATCC 33591	Salasia et al. (2004)
	Regulatory proteins	<i>ssl7-F</i>	GGT TTA TTC ATA GCG CAG TAT C	58	<i>S. aureus</i> ATCC 25923	
	Accessory gene regulator protein	<i>ssl7-R</i>	CAA CGT TTC ATC GTT AAG CTG C	58		
<i>agr</i>	Surface factors		ATG CAC ATG GTG CAC ATG CA	55	<i>S. aureus</i> ATCC 25923	Dufour et al. (2002)
	Biofilm-associated protein		CAT AAT CAT GAC GGA ACT TGC TGC GCA	57		
<i>cap5H</i>	CP5 synthesis enzyme	<i>bapF-6m</i>	ACT TAY TRC CHT ATA TCG AAR TAG	57	<i>S. xylophilus</i> ATCC 29971	Cucarella et al. (2004)
		<i>Sasp-7c</i>	GCT GTT GAA GTT AAT ACT GTA CCT GC	50	<i>S. aureus</i> NCTC 8325-4	Ote et al. (2011)
<i>cap8H</i>	CP8 synthesis enzyme	<i>cap5H</i>	ATG AGG ATA GCG ATT GAA AA	50	<i>S. aureus</i> ATCC 33591	Ote et al. (2011)
		<i>cap5R</i>	CGC TTC TTA ATC ACT TTT GC	58	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
<i>clfA</i>	Clumping factor ClfA	<i>cap8H</i>	ATC GAA GAA CAT ATC CAA GG	55	<i>S. aureus</i> ATCC 29213	Salaberry et al. (2015)
		<i>clfA-F</i>	TTC ATC ACC AAT ACC TTT TA	50	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
<i>eno</i>	Laminin binding protein	<i>clfA-R</i>	GGC AAC GAA TCA AGC TAA TAC AC	55	<i>S. aureus</i> ATCC 29213	Salaberry et al. (2015)
		<i>eno-1</i>	TTG TAC TAC CTA TGC CAG TTG TC	50	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
<i>fnbpA</i>	Fibronectin-binding protein A	<i>eno-2</i>	ACG TGC AGC AGC TGA CT	57	<i>S. aureus</i> ATCC 29213	Wang et al. (2016)
		<i>fnbpA-F</i>	CAA CAG CAT CTT CAG TAC CTT C	50	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
<i>fnbpB</i>	Fibronectin-binding protein B	<i>fnbpA-R</i>	GCG GAG ATC AAA GAC AA	50	<i>S. aureus</i> ATCC 25923	Wang et al. (2016)
		<i>fnbpB-F</i>	CAT CTA TAG CTG TGT GG	57	<i>S. aureus</i> ATCC 33591	Rohde et al. (2007)
<i>ica</i>	Polysaccharide intercellular adhesion	<i>fnbpB-R</i>	GGA GAA GGA ATT AAG GCG	57	<i>S. epidermidis</i> ATCC 35984	
		<i>icaFSe</i>	GCC GTC GCC TTG AGC GT			
		<i>icaRSe</i>	CTG TTT CAT GGA AAC TCC			
			TCG ATG CGA TTT GTT CAA ACA T			

¹F = forward; R = reverse.

Table 3. Distribution of 1,238 phenotypically different non-*aureus* staphylococci (NAS) originating from quarter milk (QM), teat apices (TA), and rectal fecal (RF) samples with mixed culture in 8 commercial dairy herds

Species	Habitat	Herd (H)																Total	
		H1		H2		H3		H4		H5		H6		H7		H8			
		N ¹	% ²	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
<i>Staphylococcus agnetis</i>	QM TA RF					1	3							3				1	1
<i>Staphylococcus arlettae</i>	QM TA RF	1	3	2	2	2	3			5	11					2	2	4	1
<i>Staphylococcus auricularis</i>	QM TA RF	1	2	1	1	1	4	2	3									1	4
<i>Staphylococcus capitis</i>	QM TA RF	8	13	3	7	4	10	23	44	3	7			2	2			41	15
<i>Staphylococcus chromogenes</i>	QM TA RF	3	9					1	1							3	2	4	1
<i>Staphylococcus cohnii</i>	QM TA RF	1	14			1	3	7	19									11	5
<i>Staphylococcus devriesei</i>	QM TA RF	11	14	12	9	5	15	11	9	4	7	29	18					83	10
<i>Staphylococcus epidermidis</i>	QM TA RF	8	25	1	2	29	50	3	9	16	30					4	4	57	23
<i>Staphylococcus equorum</i>	QM TA RF	1	14	3	3			15	13							1	1	1	4
<i>Staphylococcus fleuretii</i>	QM TA RF	4	9	17	27	1	4	8	8	1	3					8	6	22	3
<i>Staphylococcus haemolyticus</i>	QM TA RF	1	14	2	14	2	50	9	10									56	15
<i>Staphylococcus hominis</i>	QM TA RF	4	8	2	2	4	7	5	5	4	7							2	8
<i>Staphylococcus hyicus</i>	QM TA RF	60	72	8	18	10	25	2	6	26	48					31	19	56	7
<i>Staphylococcus kloosii</i>	QM TA RF	1	2	1	14													106	33
<i>Staphylococcus lentus</i>	QM TA RF	4	13															1	4
		9	16	7	6	18	30	18	19	3	6							1	1
		54	81	80	77	77	83	38	66	27	45							4	2
		2	14					1	33										
		5	8	1	1	4	15	13	13	3	6							78	9
		17	22	19	32	7	15	6	16									276	70
		1	14	8	57	1	25	2	40									3	8
																		38	5
																		49	17
																		12	31
																		2	1

Continued

Table 3 (Continued). Distribution of 1,238 phenotypically different non-*aureus* staphylococci (NAS) originating from quarter milk (QM), teat apices (TA), and rectal fecal (RF) samples with mixed culture in 8 commercial dairy herds

Species	Habitat	Herd (H)																Total	
		H1		H2		H3		H4		H5		H6		H7		H8			
		N ¹	% ²	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
<i>Staphylococcus pettenkoferi</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus rostri</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus saprophyticus</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus schleiferi</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus sciuri</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus simulans</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus succinus</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus vitulinus</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus warneri</i>	QM																		
	TA																		
	RF																		
<i>Staphylococcus xylosus</i>	QM																		
	TA																		
	RF																		

¹Number of phenotypically different (per sample) NAS isolates per habitat and herd.²Percentage of samples positive for NAS per habitat and herd (samples can harbor more than one NAS species).³In herds 6, 7, and 8, only quarter milk samples were collected.

in either the same or a different quarter, resulting in 230 clinical mastitis cases that were sampled. Twenty percent, 12%, and 8% of the quarter milk samples from healthy quarters, quarters with subclinical, or clinical signs were bacteriologically negative, respectively. Table 4 displays the percentage of samples only positive for NAS and the maximum number of NAS species per herd.

NAS Distribution. *Staphylococcus chromogenes*, *S. hominis*, and *S. xylosus* were present in all 3 types of quarter milk samples (i.e., healthy quarters, quarters with subclinical, or with clinical signs; Table 6). *Staphylococcus chromogenes* was the most prevalent species in healthy quarters or quarters with subclinical signs, and one of the 3 most commonly isolated species from quarters showing clinical signs. *Staphylococcus haemolyticus* was the second most and most frequently isolated NAS species from quarters with subclinical and quarters with clinical signs, respectively. In healthy quarters, *S. equorum*, *S. devriesei*, and *S. hominis* were equally present and thus the second most prevalent species. In quarters with subclinical signs, the third most prevalent species was *S. xylosus*. *Staphylococcus equorum* had the next highest prevalence in quarters with clinical signs. Based on the definitions according to Kalmus et al. (2013), milk samples from clinical mastitis cases harboring NAS only had score 1 (mild), 2 (moderate), or 3 (severe) in 70%, 15%, or 4% of the cases, respectively. Three milk samples originating from clinical mastitis harboring NAS only were not classified due to a lack of information on the severity score.

Quarter SCC. The NAS-positive milk samples (excluding those showing clinical signs, $n = 57$) had a geometric mean qSCC of 109,000 cells/mL of milk [interquartile range (IQR) from 52,000 to 210,000 cells/mL of milk]. Milk samples positive for a major mastitis pathogen only (*S. aureus*, *S. dysgalactiae*, *Serratia* spp., *Pasteurella multocida*, and *Trueperella pyogenes*, $n = 6$) had a geometric mean qSCC of 155,000 cells/mL of milk with a IQR from 60,000 cells/mL of milk to 830,000 cells/mL of milk, whereas samples yielding no growth ($n = 93$) had a geometric mean qSCC of 34,000 cells/mL of milk (IQR from 20,000 to 57,000 cells/mL of milk). Samples positive for *Corynebacterium* spp. ($n = 60$) had a geometric mean qSCC of 53,000 cells/mL (IQR from 24,000 to 101,000 cells/mL of milk). The NAS-positive milk samples (all species combined) had a significantly higher LnqSCC than the milk samples yielding no growth ($P = 0.000$) and *Corynebacterium* spp. ($P = 0.008$), yet the LnqSCC was not different from the major pathogen-positive milk samples. The geometric mean qSCC of samples positive for *S. chromogenes* ($n = 20$) or *S. haemolyticus* ($n = 10$) only were 156,000 cells/mL of milk (IQR from

Table 4. Samples positive for non-*aureus* staphylococci (NAS) allowing for other pathogens to be present in the 3 habitats quarter milk, teat apices, and rectal feces (“Overall distribution”) and samples positive for NAS only in the 3 milk strata (“Virulence potential in milk strata”) in commercial dairy herds

Item	N _{total} samples	N _{NAS} isolates	Range _{NAS} species/sample	% NAS-positive samples								Maximum number of different NAS species										
				Herd (H)								Herd (H)								Total		
				H1	H2	H3	H4	H5	H6	H7	H8	Total	H1	H2	H3	H4	H5	H6	H7	H8	Total	
Overall distribution																						
Habitat																						
Quarter milk	803	375	1-4	44	31	42	50	21	28	25	18	33	9	12	7	14	5	6	15	8	22	22
Teat apices	192	830	1-8	100	100	95	97	98	— ¹	—	—	98	15	13	12	14	8	—	—	—	22	22
Rectal feces	80	33	1-3	55	35	19	31	6	—	—	—	28	7	5	2	4	1	—	—	—	12	12
Total	1,075	1,238	1-8	63	48	57	58	40	29	28	28	44	16	15	13	17	10	6	15	8	25	25
Virulence potential in milk strata																						
Quarter milk stratum																						
Healthy quarter	312	17	1-2	0	0	0	22	2	2	4	8	4	0	0	0	4	1	1	3	4	8	8
Subclinical signs	261	74	1-4	13	14	4	31	0	26	6	19	16	3	2	1	7	0	3	1	2	8	8
Clinical signs	230	43	1-3	18	25	—	4	6	8	13	0	12	1	6	—	3	3	2	5	0	10	10
Total	803	134	1-4	10	13	2	22	3	12	9	10	10	4	6	1	10	4	3	8	5	15	15

¹In herds 6, 7, and 8, only quarter milk samples were collected.

Table 5. Distribution of 833 phenotypically different non-*aureus* staphylococci (NAS) on teat apices originating from lactating cows (n = 15), sampled before and after milking, and from dry cows (n = 18) in 5 commercial dairy herds

NAS species	Teat apex strata	Herd (H)												Total	
		H1		H2		H3		H4		H5					
		N ¹	% ²	N	%	N	%	N	%	N	%	N	%	N	%
<i>Staphylococcus agnetis</i>	Before milking					1	6					1	2		
	After milking														
	Dry cows			3	17	2	6+					2	13	7	8
<i>Staphylococcus arlettae</i>	Before milking			2	17							1	6	3	5
	After milking											2	17	3	4
<i>Staphylococcus auricularis</i>	Dry cows	1	6												
	Before milking	2	13	1	8	2	13	7	50			1	6	13	15
	After milking					2	13	1	13			1	6	4	7
<i>Staphylococcus capitis</i>	Dry cows	6	19	2	10			15	56			1	8	24	21
	Before milking					1	6	5	50			6	8	6	8
	After milking							2	25			3	5	3	5
<i>Staphylococcus chromogenes</i>	Dry cows	1	13									2	3	2	3
	Before milking	2	13												
	After milking	1	13			9	50	1	13	7	38	18	27	18	27
<i>Staphylococcus cohnii</i>	Before milking	2	25			4	25			4	19	10	15	10	15
	After milking	5	31	1	5	16	100	2	13	5	33	29	28	29	28
	Dry cows														
	Before milking			18	75										
	After milking			16	75										
<i>Staphylococcus devriesei</i>	Dry cows			32	80			2	13			16	15	18	15
	Before milking			5	33	1	6	15	75			34	25	34	25
	After milking							12	50			21	18	21	18
<i>Staphylococcus epidermidis</i>	Dry cows	1	6	4	15			18	56			12	7	12	7
	Before milking											23	18	23	18
	After milking														
<i>Staphylococcus equorum</i>	Dry cows	3	25	17	60			1	13			4	5	4	5
	Before milking	1	6									18	18	18	18
	After milking	11	63	4	33	6	38	1	13	12	69	34	45	34	45
<i>Staphylococcus fleuretii</i>	Before milking	8	50	2	17	4	25			9	31	23	25	23	25
	After milking	41	88	2	10			1	6	5	42	49	31	49	31
	Dry cows														
	Before milking														
	After milking														
<i>Staphylococcus haemolyticus</i>	Dry cows	4	25									4	6		
	Before milking	24	100	20	92	33	94	8	50	8	44	93	75	93	75
	After milking	11	75	4	33	23	63	5	50	3	19	46	45	46	45
<i>Staphylococcus hominis</i>	Dry cows	19	75	56	95	21	100	25	81	16	83	137	86	137	86
	Before milking	13	63	5	25	4	19	3	25	3	25	25	22	25	22
	After milking	4	25	3	25	2	13	2	25	2	25	11	15	11	15
<i>Staphylococcus kloosii</i>	Dry cows			11	40	1	13	1	6			13	14	13	14
	Before milking			2	8							2	2	2	2
	After milking														
<i>Staphylococcus lentus</i>	Dry cows														
	Before milking	1	13									1	2	1	2
	After milking														
<i>Staphylococcus pettenkoferi</i>	Dry cows														
	Before milking														
	After milking														
	Dry cows							3	19			3	4	3	4

Continued

Table 5 (Continued). Distribution of 833 phenotypically different non-*aureus* staphylococci (NAS) on teat apices originating from lactating cows (n = 15), sampled before and after milking, and from dry cows (n = 18) in 5 commercial dairy herds

NAS species	Teat apex strata	Herd (H)									
		H1		H2		H3		H4		H5	
		N ¹	% ²	N	%	N	%	N	%	N	%
<i>Staphylococcus rostri</i>	Before milking										
	After milking										
<i>Staphylococcus saprophyticus</i>	Dry cows										
	Before milking										
<i>Staphylococcus sciuri</i>	After milking										
	Dry cows										
<i>Staphylococcus simulans</i>	Before milking										
	After milking										
<i>Staphylococcus succinus</i>	Dry cows										
	Before milking										
<i>Staphylococcus vitulinus</i>	After milking										
	Dry cows										
<i>Staphylococcus xyloso</i>	Before milking										
	After milking										
<i>Staphylococcus xyloso</i>	Dry cows										
	Before milking										
<i>Staphylococcus xyloso</i>	After milking										
	Dry cows										

¹Number of phenotypically different (per sample) NAS isolates per teat apex stratum and herd.
²Percentage of sampled teat apices positive for a NAS species per teat apex strata and herd (teat apices can harbor more than 1 NAS species).

Table 6. Distribution of samples positive for only non-*aureus* staphylococci (NAS) in the 3 quarter milk strata: healthy quarters, quarters with subclinical, and clinical signs in commercial dairy herds and the presence of selected virulence and methicillin-resistance genes in NAS originating from healthy quarters and quarters with clinical signs¹

NAS species	Quarter milk						Total
	≤50,000 cells/mL		>50,000 cells/mL		Clinical signs		
	N ²	% ³	N	%	N	%	
<i>Staphylococcus chromogenes</i>	2	8	18	72	5 ⁴	20	25
<i>Staphylococcus haemolyticus</i>			10	67	5 ^{5,6}	33	15
<i>Staphylococcus equorum</i>	3 ⁵	43			4 ^{5,6}	57	7
<i>Staphylococcus hominis</i>	2	40	1	20	2	40	5
<i>Staphylococcus epidermidis</i>			1	25	3 ^{6,7}	75	4
<i>Staphylococcus xylosus</i>			4	100			4
<i>Staphylococcus devriesei</i>	3	100					3
<i>Staphylococcus simulans</i>					2 ⁶	100	2
<i>Staphylococcus auricularis</i>			1	100			1
<i>Staphylococcus capitis</i>	1 ⁶	100					1
<i>Staphylococcus cohnii</i>	1	100					1
<i>Staphylococcus arlettae</i> / <i>S. xylosus</i>	1	100					1
<i>S. chromogenes</i> / <i>S. epidermidis</i>			1	100			1
<i>S. chromogenes</i> / <i>S. equorum</i> ⁵					1 ⁵	100	1
<i>S. chromogenes</i> / <i>S. haemolyticus</i> ⁶					1 ⁶	100	1
<i>S. chromogenes</i> / <i>S. hominis</i>	1	100					1
<i>S. chromogenes</i> / <i>Staphylococcus sciuri</i>			1	100			1
<i>S. devriesei</i> / <i>S. haemolyticus</i>			1	100			1
<i>S. devriesei</i> / <i>S. hominis</i>			1	100			1
<i>S. devriesei</i> / <i>S. xylosus</i>			1	100			1
<i>S. epidermidis</i> / <i>S. haemolyticus</i>			1	100			1
<i>S. haemolyticus</i> / <i>S. hominis</i>			1	100			1
<i>S. sciuri</i> ⁶ / <i>S. xylosus</i> ^{5,6}					1 ^{5,6}	100	1
<i>Staphylococcus succinus</i> ^{6,7} / <i>S. xylosus</i> ⁶					1 ^{6,7}	100	1
<i>S. equorum</i> ⁵ / <i>S. haemolyticus</i> ⁶ / <i>Staphylococcus lentus</i> ⁶					1 ^{5,6}	100	1
<i>S. equorum</i> ⁵ / <i>S. haemolyticus</i> ⁶ / <i>S. xylosus</i> ⁵					1 ^{5,6}	100	1
<i>S. auricularis</i> / <i>S. devriesei</i> / <i>S. haemolyticus</i> / <i>S. hominis</i>			1	100			1
Total	14	17	43	51	27	32	84

¹No other bacteria were grown from those quarter milk samples.

²Number of quarters positive for 1 or more NAS species.

³Percentage of (the combination of) NAS species, present in healthy quarter milk samples, samples from quarters with subclinical or with clinical signs.

⁴Samples harboring at least 1 NAS isolate positive for the virulence gene *cap5H*.

⁵Samples harboring at least 1 NAS isolate positive for the virulence gene *bap*.

⁶Samples harboring at least 1 NAS isolate positive for the methicillin-resistance gene *mecA*.

⁷Samples harboring at least 1 NAS isolate positive for the virulence gene *agrA*.

81,000 to 212,000 cells/mL of milk) and 177,000 cells/mL of milk (IQR from 78,000 to 238,000 cells/mL of milk), respectively. These milk samples had a higher LnqSCC than bacteriologically negative milk samples ($P = 0.000$ and $P = 0.001$, respectively) and samples harboring *Corynebacterium* spp. ($P = 0.008$ and $P = 0.049$, respectively), yet the LnqSCC was not different from the major pathogen-positive milk samples.

Distribution of Virulence Genes. Table 7 outlines the results of NAS isolates positive for the virulence genes *agrA*, *bap*, and *cap5H* and methicillin-resistance gene *mecA*. At least one of those 4 genes was found in 71% of the 43 NAS isolates originating from quarters with signs of clinical mastitis, whereas only 25% of the NAS isolates from quarters with a qSCC ≤50,000 cells/mL were harboring either *bap* or *mecA*. Other viru-

lence genes and methicillin-resistance genes were either absent in all tested isolates (*cap8H*, *clfA*, *coa*, *fnbpA*, *fnbpB*, *hla*, *hly*, *ica*, *mecC*, *nuc*, *ssl7*, and *vwb*) or present in all tested isolates (*eno*).

DISCUSSION

This study aimed at extending our knowledge of the species-specific NAS presence and distribution in bovine quarter milk, on teat apices, and in rectal feces. The presence of NAS in rectal feces, reported recently by our group for the first time (Wuytack et al., 2019), was confirmed. Actually, 28% of the cows, ranging from 6% to 55% per herd, were shedding NAS in the feces. Moreover, we were able to study the presence of NAS over the 3 habitats in detail, expanding our knowledge

on the ecology of this group of staphylococci. Nine species, namely *S. arlettae*, *S. auricularis*, *S. chromogenes*, *S. cohnii*, *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. hominis*, and *S. vitulinus*, were present in all habitats in the same herds. In addition, the virulence potential of NAS was evaluated by comparing their distribution in milk samples originating from healthy quarters and quarters with signs of clinical mastitis, and by determining the presence of selected virulence genes.

This is not the first species-specific study that focuses on the distribution of NAS in quarter milk and on teat apices (De Visscher et al., 2016a; Mahmmoud et al., 2018), yet we also included teat apex samples from both lactating and dry cows and rectal feces as a potential NAS source. The same species from rectal feces were also recovered from either quarter milk or teat apices, or both, in the same herds. These NAS species were further subtyped with the use of the strain-typing technique, random amplification of polymorphic DNA (RAPD)-PCR. For some species, the same RAPD type was found in rectal feces, on teat apices, and in quarter milk originating from the same herd, indicating that fecal NAS are a potential cause of NAS IMI (Wuytack et al., 2020).

Non-*aureus* staphylococci were recovered from 33% of the quarter milk samples, including samples that also yielded other pathogens, which is comparable to other studies (Supré et al., 2011; Young et al., 2015; Condas et al., 2017b), but mixed growth samples with 2 different species were not included in the study of Condas et al. (2017b). In heifers, the reported prevalence of NAS in milk is even higher (Piepers et al., 2010; De Visscher et al., 2016a) and lower NAS prevalence in milk samples, ranging from 6% to 13%, has also been

reported (Paradis et al., 2010; Piessens et al., 2011; Mørk et al., 2012). Still, the large variation in study criteria (number of colonies, pure versus mixed culture) and identification methods between studies hampers meaningful comparison. Systematic sampling of quarter milk, teat apices, and rectal feces was used because it was the more practical way on farm. Bias might have been introduced if the factor under study was related to the sampling interval (Dohoo et al., 2014), which we believe was not the case. The 5 most prevalent species in this study were *S. chromogenes*, *S. haemolyticus*, *S. equorum*, *S. hominis*, and *S. cohnii*. Other studies also found a high prevalence of *S. epidermidis*, *S. simulans*, and *S. xylosus* (Sampimon et al., 2009a; Condas et al., 2017b; Jenkins et al., 2019).

The prevalence of NAS-positive teat apices is high in our study. The teat apices of dry cows all harbored NAS (1–8 species), which is comparable to the findings of Adkins et al. (2018a). Of the lactating cows, 94% of the teat apices were positive for NAS (also 1–8 species), which is similar to what Braem et al. (2013) reported. Other studies reported a lower prevalence of NAS on teat apices, ranging from 59.3% to 87% (De Visscher et al., 2014, 2016b; Mahmmoud et al., 2018). The NAS isolates belonged to 22 different species, with a clear variation in distribution among herds, a finding that has been observed by others (De Visscher et al., 2016b; Adkins et al., 2018a). The predominant species on teats in our study were *S. haemolyticus*, *S. equorum*, and *S. cohnii*. These findings are in alignment with other reports (Braem et al., 2013; De Visscher et al., 2014; Mahmmoud et al., 2018), although *S. cohnii* was mostly present on one herd (De Visscher et al., 2014) and on the teat apices before the milking process (De Visscher

Table 7. Distribution of virulence genes *agrA*, *bap*, and *cap5H*, and the methicillin-resistance gene *mecA* in 59 non-*aureus* staphylococci (NAS) isolates originating from quarter milk from healthy quarters (n = 16) and quarters with clinical signs (n = 43)

NAS species	N _{isolates}	<i>agrA</i>	<i>bap</i>	<i>cap5H</i>	<i>mecA</i>	N _{pos. isolates} ¹	% _{pos. isolates} ²
SCC ≤50,000 cells/mL	16						
<i>Staphylococcus capitis</i>	1				1	1	100
<i>Staphylococcus equorum</i>	3		3			3	100
Subtotal			3		1	4	25
Clinical signs	43						
<i>Staphylococcus chromogenes</i>	8			2		2	25
<i>Staphylococcus epidermidis</i>	4	4			4	4	100
<i>S. equorum</i>	9		9		2	9	100
<i>Staphylococcus haemolyticus</i>	11		1		9	9	82
<i>Staphylococcus lentus</i>	1				1	1	100
<i>Staphylococcus sciuri</i>	1				1	1	100
<i>Staphylococcus simulans</i>	2				1	1	50
<i>Staphylococcus succinus</i>	1	1			1	1	100
<i>Staphylococcus xylosus</i>	3		2		2	3	100
Subtotal		5	12	2	21	31	72
Total	59	5	15	2	22	35	59

¹Number of NAS isolates positive for *agrA*, *bap*, *cap5H*, or *mecA* (or a combination of these).

²Percentage of NAS isolates positive for *agrA*, *bap*, *cap5H*, or *mecA* (or a combination of these).

et al., 2016b). *Staphylococcus simulans* was only isolated from the teat apices of dry cows, which is corroborated by the results of Mahmmod et al. (2018). Other species that were only isolated from dry cows were *S. pettenkoferi*, *S. fleurettii*, and *S. rostri*. A notable difference in the number of NAS isolates before ($n = 280$) and after the milking process ($n = 155$) on the teat apices supports the findings of De Visscher et al. (2014), suggesting that NAS are washed away during the milking process or susceptible to the premilking disinfectant products (when used). Still, *S. epidermidis* was only isolated after the milking process. As *S. epidermidis* is regularly isolated from infected quarters, cross-infection via the milk machine liners could have been occurred. Milkers' skin is also known as an infection source for NAS IMI (Thorberg et al., 2006; Piessens et al., 2011; De Visscher et al., 2014).

At least one dairy cow in every herd was shedding NAS species in feces, where 28% of the rectal feces samples were NAS-positive. The latter was lower than what we reported earlier (52%; Wuytack et al., 2019), yet in that study we only sampled cows (repeatedly) from one herd. *Staphylococcus rostri* was only identified in quarter milk and on teat apices in the present study but not in rectal feces, whereas it was the most prevalent species in our previous study. This suggests a herd-specific distribution of NAS in feces exists as well, as is true for NAS from quarter milk and teat apices (Piessens et al., 2011; De Visscher et al., 2016b). The NAS species originating from rectal feces were also found both on teat apices and in quarter milk in the same herd, as aforementioned. Strain-typing NAS, present in the 3 habitats on the same herds, revealed that the same RAPD type was present in each of them for some species. These findings suggest that fecal NAS can infect the bovine mammary gland (Wuytack et al., 2020).

The occurrence of NAS in milk samples (10%) was similar to another study where NAS recovery in pure culture was studied, not taking into account samples yielding other pathogens (Paradis et al., 2010) but dissimilar to the study of Condas et al. (2017b), with a NAS prevalence of 26% in pure culture. The difference in study prevalence of NAS could be explained by the use of a semi-selective agar, MSA, in our study. This agar improves the differentiation of phenotypically different colonies from NAS (De Visscher et al., 2013) just as we observed in the current study, but we also observed better growth on MSA than on esculin blood agar of certain *Bacillus* spp. and *Corynebacterium* spp. We corroborated that *S. chromogenes*, *S. haemolyticus*, and *S. xylosum* are 3 of the 5 most prevalent species causing bovine IMI (Vanderhaeghen et al., 2014; Condas et al., 2017a), yet *S. equorum* and *S. hominis* completed

the top 5 most isolated species. *Staphylococcus simulans* was isolated only 2 times in our study, unlike previous studies (Mørk et al., 2012; Nyman et al., 2018).

The NAS as a group was found in 12% of the quarter milk samples originating from clinical mastitis cases, comparable to other studies that report a NAS prevalence in clinical mastitis cases of 5% ranging up to 20% (Persson Waller et al., 2011; Verbeke et al., 2014; Levison et al., 2016). The NAS-positive samples from clinical mastitis cases range from 0% in herd 8 to 25% in herd 2. This difference between herds might be explained by the variation in NAS species and their virulence potential. Herd-specific factors (e.g., housing conditions, clipping of the udder, and milking protocol) also influence species-specific prevalence as well as seasonal effects, although more research is required to identify species-specific risk factors (De Visscher et al., 2016a, 2017; Dolder et al., 2017).

Intramammary infection with NAS as a group caused a moderate elevation in qSCC. The elevation in qSCC (109,000 cells/mL of milk) is situated between the geometric mean of 70,000 cells/mL found by Condas et al. (2017a) and 137,000 cells/mL of milk reported by Supré et al. (2011). This variation in qSCC could be explained by the difference in NAS distribution because the level of elevation in SCC is species specific (Fry et al., 2014; Nyman et al., 2018). The NAS-positive milk samples (all species combined) had a significantly higher LnqSCC than the milk samples yielding no growth and *Corynebacterium* spp. The qSCC was not different from quarter milk with major mastitis pathogens, suggesting that NAS as a group have some effect on udder health. Still, only 6 quarters were major pathogen positive, reducing the power of finding true differences. Furthermore, some NAS species tend to be more relevant for the udder health because samples harboring NAS species other than *S. chromogenes* or *S. haemolyticus* only did not have a significantly higher LnqSCC than samples yielding no growth or *Corynebacterium* spp. (data not shown). This finding is in alignment with other studies (De Visscher et al., 2015; Condas et al., 2017a; Adkins et al., 2018b).

Virulence genes *agrA*, *bap*, and *cap5H* were found in 44% of the NAS isolates originating from quarters with clinical signs, which is in contrast to healthy quarters of which only 19% were harboring *bap*. Naushad et al. (2019) investigated the profile of 191 virulence factors in NAS, based on whole-genome sequencing, and found distinctive patterns of associations for low qSCC (defined as <150,000 cells/mL in that study) and clinical mastitis isolates. This finding is in alignment with our results, although we only tested a limited number of virulence genes ($n = 15$). Despite the relatively high number of phenotypically identified *Staphylococcus* spp.

isolates ($n = 1,676$) in our study, only a limited number of molecularly identified NAS ($n = 134$) were present in quarter milk samples harboring NAS only. In addition to the limited number of NAS isolates, they belonged to several NAS species ($n = 15$) divided over the 3 different milk strata, leaving an insufficient number for species-specific statistical analysis regarding the virulence potential.

All isolates tested positive for the biofilm-related gene, *eno*, a finding that is comparable to other studies that also reported a prevalence as high as 88% and 92.6%, respectively (Darwish and Asfour, 2013; Srednik et al., 2017). In our study, the prevalence of the *mecA* gene in NAS isolates from clinical mastitis cases was 49%, situated between the *mecA* prevalence of 29% described by Khazandi et al. (2018) and the 95% reported by Mahato et al. (2017). Still, Khazandi et al. (2018) tested only oxacillin-resistant isolates instead of all NAS isolates originating from clinical mastitis cases, as in our study, and might have missed oxacillin-susceptible *mecA*-positive staphylococci (Mahato et al., 2017). It is remarkable that *mecA* was found in 49% of the NAS isolates originating from clinical mastitis and only in one isolate (6%) originating from healthy quarters. Although the presence of certain antimicrobial genes may be associated with reduced fitness of the strain, the methicillin-resistance gene, *mecA* in bovine NAS isolates might be linked with virulence genes or pathogenicity islands, supposedly both present on the mobile element, *SCCmec*. The presence of virulence genes and the methicillin-resistance gene, *mecA*, dovetails nicely with the prevalence of NAS in the opposite strata of the quarter milk because NAS species were recovered 3 times as frequently in samples from clinical mastitis cases compared with milk samples from healthy quarters, suggesting that some NAS isolates have the ability to cause (mild) clinical mastitis.

CONCLUSIONS

This study reaffirms the presence of NAS in rectal feces from dairy cows in every sampled herd, with a range of 6% to 55% of the cows shedding NAS. Nine species, namely *S. arlettae*, *S. auricularis*, *S. chromogenes*, *S. cohnii*, *S. devriesei*, *S. equorum*, *S. haemolyticus*, *S. hominis*, and *S. vitulinus*, were present in quarter milk, teat apices, and rectal feces in the same herds. Further research should investigate the potential of fecal NAS as a mammary gland infection source or NAS teat apex colonization, or both. Furthermore, our findings suggest that NAS have the ability to cause mild clinical mastitis because the occurrence of those NAS, in milk samples harboring NAS only, was larger in those originating from clinical mastitis (12%) than

the occurrence in milk samples from healthy quarters (4%). Studying the distribution of virulence genes in a selection of NAS isolates from 2 contrasting milk sample strata strengthened this finding.

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REFERENCES

- Adkins, P. R. F., S. Dufour, J. N. Spain, M. J. Calcutt, T. J. Reilly, G. C. Stewart, and J. R. Middleton. 2018a. Cross-sectional study to identify staphylococcal species isolated from teat and inguinal skin of different-aged dairy heifers. *J. Dairy Sci.* 101:3213–3225. <https://doi.org/10.3168/jds.2017-13974>.
- Adkins, P. R. F., S. Dufour, J. N. Spain, M. J. Calcutt, T. J. Reilly, G. C. Stewart, and J. R. Middleton. 2018b. Molecular characterization of non-*aureus* *Staphylococcus* spp. from heifer intramammary infections and body sites. *J. Dairy Sci.* 101:5388–5403. <https://doi.org/10.3168/jds.2017-13910>.
- Barbano, D. M., Y. Ma, and M. V. Santos. 2006. Influence of raw milk quality on fluid milk shelf life. *J. Dairy Sci.* 89:E15–E19. [https://doi.org/10.3168/jds.S0022-0302\(06\)72360-8](https://doi.org/10.3168/jds.S0022-0302(06)72360-8).
- Bjerketorp, J., M. Nilsson, A. Ljungh, J. I. Flock, K. Jacobsson, and L. Frykberg. 2002. A novel von Willebrand factor binding protein expressed by *Staphylococcus aureus*. *Microbiology* 148:2037–2044. <https://doi.org/10.1099/00221287-148-7-2037>.
- Braem, G., S. De Vliegher, B. Verbist, V. Piessens, E. Van Coillie, L. De Vuyst, and F. Leroy. 2013. Unraveling the microbiota of teat apices of clinically healthy lactating dairy cows, with special emphasis on coagulase-negative staphylococci. *J. Dairy Sci.* 96:1499–1510. <https://doi.org/10.3168/jds.2012-5493>.
- Cameron, M., H. W. Barkema, J. De Buck, S. De Vliegher, M. Chaffer, J. Lewis, and G. P. Keefe. 2017. Identification of bovine-associated coagulase-negative staphylococci by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a direct transfer protocol. *J. Dairy Sci.* 100:2137–2147. <https://doi.org/10.3168/jds.2016-12020>.
- Cameron, M., J. Perry, J. R. Middleton, M. Chaffer, J. Lewis, and G. P. Keefe. 2018. Short communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. *J. Dairy Sci.* 101:590–595. <https://doi.org/10.3168/jds.2017-13226>.
- Condas, L. A. Z., J. De Buck, D. B. Nobrega, D. A. Carson, S. Nushad, S. De Vliegher, R. N. Zadoks, J. R. Middleton, S. Dufour, J. P. Kastelic, and H. W. Barkema. 2017b. Prevalence of non-*aureus* staphylococci species causing intramammary infections in Canadian dairy herds. *J. Dairy Sci.* 100:5592–5612. <https://doi.org/10.3168/jds.2016-12478>.
- Condas, L. A. Z., J. De Buck, D. B. Nobrega, D. A. Carson, J. P. Roy, G. P. Keefe, T. J. DeVries, J. R. Middleton, S. Dufour, and H. W. Barkema. 2017a. Distribution of non-*aureus* staphylococci species in udder quarters with low and high somatic cell count, and clinical

- cal mastitis. J. Dairy Sci. 100:5613–5627. <https://doi.org/10.3168/jds.2016-12479>.
- Cucarella, C., M. A. Tormo, C. Ubeda, M. P. Trotonda, M. Monzon, C. Peris, B. Amorena, I. Lasa, and J. R. Penades. 2004. Role of biofilm-associated protein *bap* in the pathogenesis of bovine *Staphylococcus aureus*. Infect. Immun. 72:2177–2185. <https://doi.org/10.1128/IAI.72.4.2177-2185.2004>.
- Darwish, S. F., and H. A. Asfour. 2013. Investigation of biofilm forming ability in Staphylococci causing bovine mastitis using phenotypic and genotypic assays. ScientificWorldJournal 2013:378492. <https://doi.org/10.1155/2013/378492>.
- De Visscher, A., F. Haesebrouck, S. Piepers, W. Vanderhaeghen, K. Supré, F. Leroy, E. Van Coillie, and S. De Vliegher. 2013. Assessment of the suitability of mannitol salt agar for growing bovine-associated coagulase-negative staphylococci and its use under field conditions. Res. Vet. Sci. 95:347–351. <https://doi.org/10.1016/j.rvsc.2013.05.015>.
- De Visscher, A., S. Piepers, F. Haesebrouck, and S. De Vliegher. 2016a. Intramammary infection with coagulase-negative staphylococci at parturition: Species-specific prevalence, risk factors, and effect on udder health. J. Dairy Sci. 99:6457–6469. <https://doi.org/10.3168/jds.2015-10458>.
- De Visscher, A., S. Piepers, F. Haesebrouck, and S. De Vliegher. 2016b. Teat apex colonization with coagulase-negative *Staphylococcus* species before parturition: Distribution and species-specific risk factors. J. Dairy Sci. 99:1427–1439. <https://doi.org/10.3168/jds.2015-10326>.
- De Visscher, A., S. Piepers, F. Haesebrouck, K. Supré, and S. De Vliegher. 2017. Coagulase-negative *Staphylococcus* species in bulk milk: Prevalence, distribution, and associated subgroup- and species-specific risk factors. J. Dairy Sci. 100:629–642. <https://doi.org/10.3168/jds.2016-11476>.
- De Visscher, A., S. Piepers, K. Supré, F. Haesebrouck, and S. De Vliegher. 2015. Short communication: Species group-specific predictors at the cow and quarter level for intramammary infection with coagulase-negative staphylococci in dairy cattle throughout lactation. J. Dairy Sci. 98:5448–5453. <https://doi.org/10.3168/jds.2014-9088>.
- De Visscher, A., K. Supré, F. Haesebrouck, R. N. Zadoks, V. Piesens, E. Van Coillie, S. Piepers, and S. De Vliegher. 2014. Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. Vet. Microbiol. 172:466–474. <https://doi.org/10.1016/j.vetmic.2014.06.011>.
- De Vliegher, S., G. Opsomer, A. Vanrolleghem, L. A. Devriese, O. C. Sampimon, J. Sol, H. W. Barkema, F. Haesebrouck, and A. de Kruif. 2004. *In vitro* growth inhibition of major mastitis pathogens by *Staphylococcus chromogenes* originating from teat apices of dairy heifers. Vet. Microbiol. 101:215–221. <https://doi.org/10.1016/j.vetmic.2004.03.020>.
- Diep, B. A., G. G. Stone, L. Basuino, C. J. Graber, A. Miller, S. A. des Etages, A. Jones, A. M. Palazzo-Ballance, F. Perdreau-Remington, G. F. Sensabaugh, F. R. DeLeo, and H. F. Chambers. 2008. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. 197:1523–1530. <https://doi.org/10.1086/587907>.
- Dohoo, I., W. Martin, and H. Stryhn. 2014. Veterinary Epidemiologic Research. 2nd ed. VER Inc., Charlottetown, Canada.
- Dolder, C., B. H. P. van den Borne, J. Traversari, A. Thomann, V. Perreten, and M. Bodmer. 2017. Quarter- and cow-level risk factors for intramammary infection with coagulase-negative staphylococci species in Swiss dairy cows. J. Dairy Sci. 100:5653–5663. <https://doi.org/10.3168/jds.2016-11639>.
- Dufour, P., S. Jarraud, F. Vandenesch, T. Greenland, R. P. Novick, M. Bes, J. Etienne, and G. Lina. 2002. High genetic variability of the *agr* locus in *Staphylococcus* species. J. Bacteriol. 184:1180–1186. <https://doi.org/10.1128/jb.184.4.1180-1186.2002>.
- Fry, P. R., J. R. Middleton, S. Dufour, J. Perry, D. Scholl, and I. Dohoo. 2014. Association of coagulase-negative staphylococcal species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. J. Dairy Sci. 97:4876–4885. <https://doi.org/10.3168/jds.2013-7657>.
- García-Alvarez, L., M. T. Holden, H. Lindsay, C. R. Webb, D. F. Brown, M. D. Curran, E. Walpole, K. Brooks, D. J. Pickard, C. Teale, J. Parkhill, S. D. Bentley, G. F. Edwards, E. K. Girvan, A. M. Kearns, B. Pichon, R. L. Hill, A. R. Larsen, R. L. Skov, S. J. Peacock, D. J. Maskell, and M. A. Holmes. 2011. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: A descriptive study. Lancet Infect. Dis. 11:595–603. [https://doi.org/10.1016/S1473-3099\(11\)70126-8](https://doi.org/10.1016/S1473-3099(11)70126-8).
- Hogan, J. S., R. N. Gonzalez, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 1999. Laboratory Handbook on Bovine Mastitis. 2nd edition ed. National Mastitis Council, Madison, WI.
- Jenkins, S. N., E. Okello, P. V. Rossitto, T. W. Lehenbauer, J. Champagne, M. C. T. Penedo, A. G. Arruda, S. Godden, P. Rapnicki, P. J. Gorden, L. L. Timms, and S. S. Aly. 2019. Molecular epidemiology of coagulase-negative *Staphylococcus* species isolated at different lactation stages from dairy cattle in the United States. PeerJ 7:e6749. <https://doi.org/10.7717/peerj.6749>.
- Kalmus, P., H. Simojoki, S. Pyörälä, S. Taponen, J. Holopainen, and T. Orro. 2013. Milk haptoglobin, milk amyloid A, and N-acetyl-beta-D-glucosaminidase activity in bovines with naturally occurring clinical mastitis diagnosed with a quantitative PCR test. J. Dairy Sci. 96:3662–3670. <https://doi.org/10.3168/jds.2012-6177>.
- Khazandi, M., A. A. Al-Farha, G. W. Coombs, M. O'Dea, S. Pang, D. J. Trott, R. R. Aviles, F. Hemmatzadeh, H. Venter, A. D. Ogunniyi, A. Hoare, S. Abraham, and K. R. Petrovski. 2018. Genomic characterization of coagulase-negative staphylococci including methicillin-resistant *Staphylococcus sciuri* causing bovine mastitis. Vet. Microbiol. 219:17–22. <https://doi.org/10.1016/j.vetmic.2018.04.004>.
- Leitner, G., R. Eligulashvily, O. Krifucks, S. Perl, and A. Saran. 2003. Immune cell differentiation in mammary gland tissues and milk of cows chronically infected with *Staphylococcus aureus*. J. Vet. Med. B Infect. Dis. Vet. Public Health 50:45–52. <https://doi.org/10.1046/j.1439-0450.2003.00602.x>.
- Levison, L. J., E. K. Miller-Cushon, A. L. Tucker, R. Bergeron, K. E. Leslie, H. W. Barkema, and T. J. DeVries. 2016. Incidence rate of pathogen-specific clinical mastitis on conventional and organic Canadian dairy farms. J. Dairy Sci. 99:1341–1350. <https://doi.org/10.3168/jds.2015-9809>.
- Lugsomya, K., P. Tummaruk, D. J. Hampson, and N. Prapasarakul. 2012. Development of a modified selective medium to enhance the recovery rate of *Brachyspira hyodysenteriae* and other porcine intestinal spirochaetes from faeces. Lett. Appl. Microbiol. 54:330–335. <https://doi.org/10.1111/j.1472-765X.2012.03213.x>.
- Mahato, S., H. U. Mistry, S. Chakraborty, P. Sharma, R. Saravanan, and V. Bhandari. 2017. Identification of variable traits among the methicillin resistant and sensitive coagulase negative staphylococci in milk samples from mastitic cows in India. Front. Microbiol. 8:1446. <https://doi.org/10.3389/fmicb.2017.01446>.
- Mahmmod, Y. S., I. C. Klaas, L. Svennesen, K. Pedersen, and H. Ingmer. 2018. Communications of *Staphylococcus aureus* and non-aureus *Staphylococcus* species from bovine intramammary infections and teat apex colonization. J. Dairy Sci. 101:7322–7333. <https://doi.org/10.3168/jds.2017-14311>.
- Mahmoudi, H., M. R. Arabestani, S. F. Mousavi, and M. Y. Alikhani. 2017. Molecular analysis of the coagulase gene in clinical and nasal carrier isolates of methicillin-resistant *Staphylococcus aureus* by restriction fragment length polymorphism. J. Glob. Antimicrob. Resist. 8:41–45. <https://doi.org/10.1016/j.jgar.2016.10.007>.
- Matthews, K. R., R. J. Harmon, and B. E. Langlois. 1991. Effect of naturally-occurring coagulase-negative staphylococci infections on new infections by mastitis pathogens in the bovine. J. Dairy Sci. 74:1855–1859. [https://doi.org/10.3168/jds.S0022-0302\(91\)78350-1](https://doi.org/10.3168/jds.S0022-0302(91)78350-1).
- Mørk, T., H. J. Jørgensen, M. Sunde, B. Kvitle, S. Sviland, S. Waage, and T. Tollersrud. 2012. Persistence of staphylococcal species and

- genotypes in the bovine udder. *Vet. Microbiol.* 159:171–180. <https://doi.org/10.1016/j.vetmic.2012.03.034>.
- Murakami, K., W. Minamide, K. Wada, E. Nakamura, H. Teraoka, and S. Watanabe. 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain reaction. *J. Clin. Microbiol.* 29:2240–2244. <https://doi.org/10.1128/JCM.29.10.2240-2244.1991>.
- National Mastitis Council. 2010. NMC Recommended Mastitis Control Program. <https://www.nmc-online.org/resources/>.
- Naushad, S., S. A. Naqvi, D. Nobrega, C. Luby, J. P. Kastelic, H. W. Barkema, and J. De Buck. 2019. Comprehensive virulence gene profiling of bovine non-*aureus* staphylococci based on whole-genome sequencing data. *mSystems* 4:e00098-18. <https://doi.org/10.1128/mSystems.00098-18>.
- Nyman, A. K., C. Fasth, and K. Persson Waller. 2018. Intramammary infections with different non-*aureus* staphylococci in dairy cows. *J. Dairy Sci.* 101:1403–1418. <https://doi.org/10.3168/jds.2017-13467>.
- Ote, I., B. Taminiau, J. N. Duprez, I. Dizier, and J. G. Mainil. 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. *Vet. Microbiol.* 153:285–292. <https://doi.org/10.1016/j.vetmic.2011.05.042>.
- Paradis, M. E., E. Bouchard, D. T. Scholl, F. Miglior, and J. P. Roy. 2010. Effect of nonclinical *Staphylococcus aureus* or coagulase-negative staphylococci intramammary infection during the first month of lactation on somatic cell count and milk yield in heifers. *J. Dairy Sci.* 93:2989–2997. <https://doi.org/10.3168/jds.2009-2886>.
- Pearson, L. J., J. H. Williamson, S. A. Turner, S. J. Lacy-Hulbert, and J. E. Hillerton. 2013. Peripartum infection with *Streptococcus uberis* but not coagulase-negative staphylococci reduced milk production in primiparous cows. *J. Dairy Sci.* 96:158–164. <https://doi.org/10.3168/jds.2012-5508>.
- Persson Waller, K. P., A. Aspan, A. Nyman, Y. Persson, and U. Grönlund Andersson. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Vet. Microbiol.* 152:112–116. <https://doi.org/10.1016/j.vetmic.2011.04.006>.
- Piepers, S., G. Opsomer, H. W. Barkema, A. de Kruif, and S. De Vliegher. 2010. Heifers infected with coagulase-negative staphylococci in early lactation have fewer cases of clinical mastitis and higher milk production in their first lactation than noninfected heifers. *J. Dairy Sci.* 93:2014–2024. <https://doi.org/10.3168/jds.2009-2897>.
- Piepers, S., G. Opsomer, E. Meyer, K. Demeyere, H. W. Barkema, A. de Kruif, and S. De Vliegher. 2009. Heifer and quarter characteristics associated with periparturient blood and milk neutrophil apoptosis in healthy heifers and in heifers with subclinical mastitis. *J. Dairy Sci.* 92:4330–4339. <https://doi.org/10.3168/jds.2009-2029>.
- Piepers, S., Y. H. Schukken, P. Passchyn, and S. De Vliegher. 2013. The effect of intramammary infection with coagulase-negative staphylococci in early lactating heifers on milk yield throughout first lactation revisited. *J. Dairy Sci.* 96:5095–5105. <https://doi.org/10.3168/jds.2013-6644>.
- Piessens, V., E. Van Coillie, B. Verbist, K. Supré, G. Braem, A. Van Nuffel, L. De Vuyst, M. Heyndrickx, and S. De Vliegher. 2011. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. *J. Dairy Sci.* 94:2933–2944. <https://doi.org/10.3168/jds.2010-3956>.
- Potter, T. L., C. Arndt, and A. N. Hristov. 2018. Short communication: Increased somatic cell count is associated with milk loss and reduced feed efficiency in lactating dairy cows. *J. Dairy Sci.* 101:9510–9515. <https://doi.org/10.3168/jds.2017-14062>.
- Pozzi, C., E. M. Waters, J. K. Rudkin, C. R. Schaeffer, A. J. Lohan, P. Tong, B. J. Loftus, G. B. Pier, P. D. Fey, R. C. Massey, and J. P. O’Gara. 2012. Methicillin resistance alters the biofilm phenotype and attenuates virulence in *Staphylococcus aureus* device-associated infections. *PLoS Pathog.* 8:e1002626. <https://doi.org/10.1371/journal.ppat.1002626>.
- Queck, S. Y., B. A. Khan, R. Wang, T. H. Bach, D. Kretschmer, L. Chen, B. N. Kreiswirth, A. Peschel, F. R. Deleo, and M. Otto. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. *PLoS Pathog.* 5:e1000533. <https://doi.org/10.1371/journal.ppat.1000533>.
- Rajala-Schultz, P. J., A. H. Torres, F. J. DeGraves, W. A. Gebr-eyes, and P. Patchanee. 2009. Antimicrobial resistance and genotypic characterization of coagulase-negative staphylococci over the dry period. *Vet. Microbiol.* 134:55–64. <https://doi.org/10.1016/j.vetmic.2008.09.008>.
- Reyher, K. K., D. Haine, I. R. Dohoo, and C. W. Revie. 2012. Examining the effect of intramammary infections with minor mastitis pathogens on the acquisition of new intramammary infections with major mastitis pathogens—A systematic review and meta-analysis. *J. Dairy Sci.* 95:6483–6502. <https://doi.org/10.3168/jds.2012-5594>.
- Rohde, H., E. C. Burandt, N. Siemssen, L. Frommelt, C. Burdelski, S. Wurster, S. Scherpe, A. P. Davies, L. G. Harris, M. A. Horst-kotte, J. K. Knobloch, C. Ragunath, J. B. Kaplan, and D. Mack. 2007. Polysaccharide intercellular adhesin or protein factors in bio-film accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials* 28:1711–1720. <https://doi.org/10.1016/j.biomaterials.2006.11.046>.
- Salaberry, S. R., A. B. Saidenberg, E. Zuniga, P. A. Melville, F. G. Santos, E. C. Guimaraes, F. Gregori, and N. R. Benites. 2015. Virulence factors genes of *Staphylococcus* spp. isolated from caprine subclinical mastitis. *Microb. Pathog.* 85:35–39. <https://doi.org/10.1016/j.micpath.2015.05.007>.
- Salasia, S. I., Z. Khusnan, C. Lammler, and M. Zschock. 2004. Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. *J. Vet. Sci.* 5:103–109. <https://doi.org/10.4142/jvs.2004.5.2.103>.
- Sampimon, O. C., H. W. Barkema, I. M. Berends, J. Sol, and T. J. Lam. 2009b. Prevalence and herd-level risk factors for intramammary infection with coagulase-negative staphylococci in Dutch dairy herds. *Vet. Microbiol.* 134:37–44. <https://doi.org/10.1016/j.vetmic.2008.09.010>.
- Sampimon, O. C., R. N. Zadoks, S. De Vliegher, K. Supré, F. Haesebrouck, H. W. Barkema, J. Sol, and T. J. G. M. Lam. 2009a. Performance of API Staph ID 32 and Staph-Zym for identification of coagulase-negative staphylococci isolated from bovine milk samples. *Vet. Microbiol.* 136:300–305. <https://doi.org/10.1016/j.vetmic.2008.11.004>.
- Schukken, Y. H., R. N. Gonzalez, L. L. Tikofsky, H. F. Schulte, C. G. Santisteban, F. L. Welcome, G. J. Bennett, M. J. Zurakowski, and R. N. Zadoks. 2009. CNS mastitis: Nothing to worry about? *Vet. Microbiol.* 134:9–14. <https://doi.org/10.1016/j.vetmic.2008.09.014>.
- Simojoki, H., T. Orro, S. Taponen, and S. Pyörälä. 2009. Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*. *Vet. Microbiol.* 134:95–99. <https://doi.org/10.1016/j.vetmic.2008.09.003>.
- Srednik, M. E., Y. D. N. Tremblay, J. Labrie, M. Archambault, M. Jacques, A. F. Cirelli, and E. R. Gentilini. 2017. Biofilm formation and antimicrobial resistance genes of coagulase-negative staphylococci isolated from cows with mastitis in Argentina. *FEMS Microbiol. Lett.* 364:fnx001. <https://doi.org/10.1093/femsle/fnx001>.
- Supré, K., F. Haesebrouck, R. N. Zadoks, M. Vanechoutte, S. Piepers, and S. De Vliegher. 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. *J. Dairy Sci.* 94:2329–2340. <https://doi.org/10.3168/jds.2010-3741>.
- Taponen, S., H. Simojoki, M. Haveri, H. D. Larsen, and S. Pyörälä. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Vet. Microbiol.* 115:199–207. <https://doi.org/10.1016/j.vetmic.2006.02.001>.
- Thorberg, B. M., I. Kuhn, F. M. Aarestrup, B. Brandstrom, P. Jonsson, and M. L. Danielsson-Tham. 2006. Pheno- and genotyping of *Staphylococcus epidermidis* isolated from bovine milk and human skin. *Vet. Microbiol.* 115:163–172. <https://doi.org/10.1016/j.vetmic.2006.01.013>.
- Valckenier, D., S. Piepers, A. De Visscher, R. M. Bruckmaier, and S. De Vliegher. 2019. Effect of intramammary infection with non-

- aureus staphylococci in early lactation in dairy heifers on quarter somatic cell count and quarter milk yield during the first 4 months of lactation. *J. Dairy Sci.* 102:6442–6453. <https://doi.org/10.3168/jds.2018-15913>.
- Valckenier, D., S. Piepers, A. De Visscher, and S. De Vliegheer. 2020. The effect of intramammary infection in early lactation with non-aureus staphylococci in general and *Staphylococcus chromogenes* specifically on quarter milk somatic cell count and quarter milk yield. *J. Dairy Sci.* 103:768–782. <https://doi.org/10.3168/jds.2019-16818>.
- Vanderhaeghen, W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, and S. De Vliegheer. 2014. Invited review: Effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. *J. Dairy Sci.* 97:5275–5293. <https://doi.org/10.3168/jds.2013-7775>.
- Verbeke, J., S. Piepers, K. Supré, and S. De Vliegheer. 2014. Pathogen-specific incidence rate of clinical mastitis in Flemish dairy herds, severity, and association with herd hygiene. *J. Dairy Sci.* 97:6926–6934. <https://doi.org/10.3168/jds.2014-8173>.
- Wang, D., L. Zhang, X. Zhou, Y. He, C. Yong, M. Shen, O. Szenci, and B. Han. 2016. Antimicrobial susceptibility, virulence genes, and randomly amplified polymorphic DNA analysis of *Staphylococcus aureus* recovered from bovine mastitis in Ningxia, China. *J. Dairy Sci.* 99:9560–9569. <https://doi.org/10.3168/jds.2016-11625>.
- Wuytack, A., A. De Visscher, S. Piepers, F. Boyen, F. Haesebrouck, and S. De Vliegheer. 2019. Non-aureus staphylococci in fecal samples of dairy cows: First report and phenotypic and genotypic characterization. *J. Dairy Sci.* 102:9345–9359. <https://doi.org/10.3168/jds.2019-16662>.
- Wuytack, A., A. De Visscher, S. Piepers, F. Haesebrouck, and S. De Vliegheer. 2020. Fecal non-aureus staphylococci are a potential cause of bovine intramammary infection. *Vet. Res. (Faisalabad)* 51:32. <https://doi.org/10.1186/s13567-020-00761-5>.
- Young, W., B. C. Hine, O. A. Wallace, M. Callaghan, and R. Bibiloni. 2015. Transfer of intestinal bacterial components to mammary secretions in the cow. *PeerJ* 3:e888. <https://doi.org/10.7717/peerj.888>.

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