Distribution of non-*aureus* staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis

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ABSTRACT

The effect of non-*aureus* staphylococci (NAS) in bovine mammary health is controversial. Overall, NAS intramammary infections (IMI) increase somatic cell count (SCC), with an effect categorized as mild, mostly causing subclinical or mild to moderate clinical mastitis. However, based on recent studies, specific NAS may affect the udder more severely. Some of these apparent discrepancies could be attributed to the large number of species that compose the NAS group. The objectives of this study were to determine (1) the SCC of quarters infected by individual NAS species compared with NAS as a group, culture-negative, and major pathogen-infected quarters; (2) the distribution of NAS species isolated from quarters with low SCC (<200,000 cells/mL) and high SCC (≥200,000 cells/mL), and clinical mastitis; and (3) the prevalence of NAS species across quarters with low and high SCC. A total of 5,507 NAS isolates, 3,561 from low SCC quarters, 1,873 from high SCC quarters, and 73 from clinical mastitis cases, were obtained from the National Cohort of Dairy Farms of the Canadian Bovine Mastitis Research Network. Of quarters with low SCC, high SCC, or clinical mastitis, 7.6, 18.5, and 4.3% were NAS positive, respectively. The effect of NAS IMI on SCC was estimated using mixed-effect linear regression; prevalence of NAS IMI was estimated using Bayesian analyses. Mean SCC of NAS-positive quarters was 70,000 cells/mL, which was higher than culture-negative quarters (32,000 cells/mL) and lower than major pathogen-positive quarters (129,000 to 183,000 cells/mL). Compared

with other NAS species, SCC was highest in quarters positive for *Staphylococcus capitis*, *Staphylococcus gal-linarum*, *Staphylococcus hyicus*, *Staphylococcus agnetis*, or *Staphylococcus simulans*. In NAS-positive quarters, *Staphylococcus xylosus* (12.6%), *Staphylococcus cohnii* (3.1%), and *Staphylococcus equorum* (0.6%) were more frequently isolated from quarters with low SCC than other NAS species, whereas *Staphylococcus sciuri* (14%) was most frequently isolated from clinical mastitis cases. Finally, in NAS-positive quarters, *Staphylococcus chro-mogenes*, *S. simulans*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus* were isolated with similar frequency from among low SCC and high SCC quarters and clinical mastitis cases. *Staphylococcus chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, *S. epidermidis*, *S. agnetis*, *Staphylococcus arlettae*, *S. capitis*, *S. gallina-rum*, *S. sciuri*, and *Staphylococcus warneri* were more prevalent in high than in low SCC quarters. Because the NAS are a large, heterogeneous group, considering them as a single group rather than at the species, or even subspecies level, has undoubtedly contributed to apparent discrepancies among studies as to their distribution and importance in IMI and mastitis.

Key words: mastitis, non-*aureus* staphylococci, somatic cell count, *Staphylococcus aureus*, coagulase-negative staphylococci

INTRODUCTION

Non-*aureus* staphylococci (NAS) are the most frequently isolated bacterial group from bovine IMI [e.g., Piepers et al. (2009)]. Several reports (Pitkälä et al., 2004; Tenhagen et al., 2006; Sampimon et al., 2009a) attributed the increasing prevalence of NAS to implementation of the 5-point contagious mastitis control program, developed in the mid last century in

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the United Kingdom (Neave et al., 1969). The wide adoption of the management practices included in this control program resulted in a decrease in prevalence of the contagious pathogens *Staphylococcus aureus* and particularly *Streptococcus agalactiae*, which has likely created a niche for other bacterial species such as the NAS (Barkema et al., 2009). As a group, NAS are considered minor pathogens, mostly associated with mild inflammation of the mammary gland (Schukken et al., 2009). However, NAS IMI has been associated with a lower incidence of major pathogen mastitis and higher milk production of NAS-infected heifers (Piepers et al., 2010). In contrast, reports indicate that NAS IMI negatively affects high-producing heifers, can persist throughout lactation in multiparous cows, and explains a large proportion of bulk milk SCC (BMSCC) in low BMSCC herds (De Vliegher et al., 2009; Schukken et al., 2009).

Good evidence indicates that the various species composing this group do not behave homogeneously and, therefore, can contribute to diverse epidemiological findings (Vanderhaeghen et al., 2014). The most common species isolated worldwide from bovine milk samples are *Staphylococcus chromogenes*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus*, with slight variations in overall distribution among studies and larger differences among herds (Taponen et al., 2007; Gillespie et al., 2009; Sampimon et al., 2009a; Supré et al., 2011; Fry et al., 2014). Some species that are more phylogenetically related can operate in a similar way (Calcutt et al., 2014; Naushad et al., 2016); for example, *S. chromogenes* and *Staphylococcus agnetis* mostly cause IMI in heifers, likely due to adaptations in udder microbiota (De Visscher et al., 2016). Other species such as *S. epidermidis* and *S. haemolyticus* seem to have greater ability to adapt to the udder and the environment, which can lead to re-infection or chronicity of IMI throughout lactation, mostly in multiparous cows (Piessens et al., 2012).

Both the distribution and effects on udder health associated with NAS vary. Researchers have previously concluded that NAS as a group are associated with higher SCC when compared with culture-negative quarters, mainly in heifers (Taponen et al., 2007; Schukken et al., 2009). A recent meta-analysis by Vanderhaeghen et al. (2014) concluded that the most common NAS species in dairy production worldwide are capable of increasing SCC, although species diverge in pathogenic potential regarding udder health. In the first study of NAS species isolated from bovine milk in Canadian herds, NAS species such as *S. chromogenes*, *S. epidermidis*, and *S. simulans* substantially increased SCC (Fry et al., 2014).

It is important to determine the distribution of NAS species according to IMI status, and their respective SCC distribution curve, to verify their current status as minor pathogens. Consequently, the objectives of this study were to determine the (1) distribution of NAS species in quarters with low SCC (<200,000 cells/mL), high SCC ($\geq$ 200,000 cells/mL), and clinical mastitis; (2) prevalence of NAS species across quarters with a low and high SCC; and (3) SCC of quarters infected by NAS species compared with NAS as a group, culture-negative, and major pathogen-infected quarters.

MATERIALS AND METHODS

Herds and Cows

Data and samples were collected from the National Cohort of Dairy Farms study conducted by the Canadian Bovine Mastitis Research Network (CBMRN) in 2007 and 2008. The study included 91 farms across 4 regions of Canada: the Maritime provinces (Prince Edward Island, Nova Scotia, and New Brunswick), Quebec, Ontario, and the Western provinces, represented by Alberta. The selection criteria are fully described by Reyher et al. (2011). Briefly, herds were selected to represent the provincial proportion of freestall systems to within 15% and to evenly distribute among 3 strata of BMSCC based on the last 12-mo BMSCC average, classified as high ($\geq$ 300,000 cells/mL), intermediate (<300,000 and $>$ 150,000 cell/mL), and low ($\leq$ 150,000 cells/mL). Herds enrolled also had to be composed of at least 80% lactating Holstein-Friesian cows milked twice a day, and herds had to participate in a DHI program. A total of 91 herds were included, 17 in Alberta, 27 in Ontario, 29 in Québec, and 18 in the Maritime provinces.

Sampling

Detailed sampling is described by Reyher et al. (2011). In short, 3 samplings schemes were conducted during the 2 yr of field collection, divided in 4 periods (March–May 2007, June–August 2007, January–March 2008, and June–August 2008). The first sampling scheme was composed of 10 random lactating cows and the 5 most recently calved cows in each herd. From these cows, quarter milk samples were collected at 3-wk intervals between March and May 2007. The sampling scheme was repeated in January to March 2008 and June to August 2008. During the June to August 2007 period, 7 quarter milk samplings were conducted at weekly intervals. The second set of samples was composed of 15 cows per herd sampled 2 to 4 wk before dry-off, at calving, and 2 wk after calving. The third set consisted of samples collected in each herd by the

farmer, following a standard protocol, from quarters with clinical mastitis.

Laboratory Analyses

Milk samples were cultured by streaking 10 μ L of milk on blood agar and MacConkey medium (according to standard bacteriological protocols), followed by phenotypic identification (Hogan et al., 2009). Pure cultures with ≥ 10 colonies of NAS per plate ($\geq 1,000$ cfu/mL) were stored in 2-mL individual cryovials (Nalgene, Rochester, NY) in 15% glycerol at -80°C labeled with the associated sample barcode (Reyher et al., 2011). Isolates from mixed infections (i.e., 2 phenotypically different colony types) possessing NAS as one of the isolates were not stored, and milk samples with growth of ≥ 3 species of pathogens were considered contaminated. Isolates of *S. aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Klebsiella* spp. were identified according to phenotypic and biochemical criteria described in the National Mastitis Council Laboratory Handbook (Hogan et al., 2009), and stored if a milk sample had >1 colony (>100 cfu/mL) in microbiological culture. Briefly, *S. aureus* isolates were identified as gram-positive cocci by Gram stain, a positive catalase test (Fisher Scientific Company, Nepean, ON, Canada), double-zone hemolysis on Columbia agar (Oxoid Ltd., Basingstoke, UK) with 5% sheep blood (Alere Inc., Ottawa, ON, Canada), a positive tube coagulase test, and a positive DNase test (Becton Dickinson, Sparks, MD). Gram-positive cocci with a negative catalase test were identified as *Streptococcus* spp., and submitted to CAMP, esculine, hippurate, inulin, and raffinose tests for species identification. *Escherichia coli* and *Klebsiella* species isolates were identified as gram-negative rods by Gram stain, lactose fermenters on MacConkey agar (Neogen Corp., Lansing, MI), and a negative oxidase test (Becton Dickinson). In addition, a triple-sugar iron agar slant, a urea, a citrate, and a spot-indole test were also set up for identifying gram-negative pathogens (Becton Dickinson; Oxoid Ltd.). *Klebsiella* species isolates were nonmotile, negative for indole production, produced urease, and used citrate. Milk samples without any growth in standard culture methods were considered culture-negative.

Subsequent to milk aliquoting for bacteriological analyses, the remaining milk sample was preserved with the addition of 2-bromo-2-nitropropane-1,3-diol tablet (Broad Spectrum Microtabs II, D&F Control Systems Inc., Dublin, CA). Samples were then shipped to Maritime Quality Milk at the University of Prince Edward Island (Charlottetown, PE, Canada) and kept frozen or refrigerated up to 5 d after shipment, until SCC was

determined using a Fossomatic cell counter (Fossomatic 4000 series, Foss Electric, Hillerød, Denmark).

Definition of Intramammary Infection

The CBMRN criteria for samples that were stored in the Mastitis Pathogen Culture Collection (MPCC; St-Hyacinthe, QC, Canada) were based on a single sample and included (1) an udder quarter IMI yielding $\geq 1,000$ cfu/mL of milk of NAS or other minor pathogen in pure culture; and (2) an udder quarter IMI yielding ≥ 100 cfu/mL of milk for major pathogens (*S. aureus*, *Strep. dysgalactiae*, *Strep. uberis*, *Klebsiella* spp.). Quarters without bacterial growth were classified as culture-negative quarters.

Data Set

A total of 118,335 milk samples were collected by the CBMRN from 31,941 lactations (range = 1–15 observation per lactation; mean = 3.7), 21,690 quarters (range = 1–23 observations per quarter; mean = 5.5), and 6,058 cows (range = 1–92 observations per cow; mean = 19.5). Of the 118,335 milk samples collected, 17,368 milk samples were considered unsuitable for laboratory analysis or contaminated. Out of 100,967 milk samples, a total of 6,302 NAS isolates meeting the aforementioned criterion were recorded from 3,225 quarters of 2,139 cows in 91 herds. A total of 721 of these isolates were not available from storage for further identification, leaving 5,581 NAS isolates for identification. From the 5,581 NAS isolates, 74 isolates were identified as another bacterial genus (Condas et al., 2017). Consequently, 5,507 NAS isolates were available to be included in the analyses.

For comparison, 47,996 culture-negative milk samples from 16,233 quarters of 5,108 cows were available (Reyher et al., 2011). According to our definition of IMI, 2,406 *S. aureus* were isolated from 1,152 quarters of 885 cows; 296 *Strep. dysgalactiae* from 215 quarters of 189 cows; 368 *Strep. uberis* from 253 quarters of 232 cows; and 171 *Klebsiella* spp. from 148 quarters of 135 cows. Bacteriological culture of these major pathogens was, however, not confirmed with molecular methods.

NAS Species Identification

A total of 5,581 NAS lyophilized bacterial isolates were received at University of Calgary; each vial was identified with a barcode representing the isolate identity. These isolates were cultured on agar plates containing 5% defibrinated sheep blood and then phenotypically characterized based on results of catalase and coagulase test, as well as Gram staining (Condas

et al., 2017). Extraction of DNA was done as described by Sampimon et al. (2009b). Subsequently, PCR amplification and partial sequencing of the 16S rRNA gene was used to confirm the identification of the different bacteria to the genus presumptively identified on the culture plates (Chakravorty et al., 2007). This procedure was followed by partial sequencing of *rpoB* gene to identify the NAS species (Mellmann et al., 2006). Sanger sequencing for all amplicons was performed at University of Calgary Core DNA Services, from which assembly and alignments were performed using Geneious (Biomatters, 2005–2014; <http://www.geneious.com>). Definitive species identification was performed based on $\geq 97\%$ identity to *rpoB* database sequences in National Center for Biotechnology Information (Drancourt and Raoult, 2002; Mellmann et al., 2006). For specific cases in which coverage identity was $\leq 50\%$, or high identity ($\geq 97\%$) was observed between 2 different species, further blast of whole *rpoB* gene was performed against whole-genome sequences of 450 bovine intramammary CNS isolates (Condas et al., 2017).

Statistical Analyses

Distribution of NAS Species in Low and High SCC, and Clinical Mastitis. Three categories of milk samples were created: samples with SCC $< 200,000$ cells/mL (low SCC), samples with SCC $\geq 200,000$ cells/mL (high SCC), and clinical mastitis samples. Distribution of species was then calculated for each species using the total number of observed NAS in that category as the denominator. Hence, results presented were proportions of NAS species in each of these 3 categories. Presence of a specific NAS species versus all other results was the outcome of each analysis. Sample type indicators were used to estimate differences in the logits of samples in each SCC category, where $P < 0.05$ was considered statistically significant. Three methods were used to contrast the distributions: (1) for the 7 most frequently isolated species (*S. chromogenes*, *S. xylosum*, *S. simulans*, *S. haemolyticus*, *Staphylococcus cohnii*, *Staphylococcus gallinarum*, and *Staphylococcus capitis*), a generalized linear mixed model using 2nd order penalized quasi-likelihood estimation with herd, cow, and quarter (lactation was not included as a level because of the low number of quarters with > 1 lactation as random effects at the intercept-level and the categories modeled as fixed-effects); (2) due to lower number of isolates per category and consequent non-convergence of the regression model, a similar model without the quarter random effect was used for *S. epidermidis* and *Staphylococcus sciuri*; and (3) for other NAS species with low occurrence, Fisher's exact test was used to compare the NAS species distribution

between sample categories (i.e., low SCC, high SCC, and clinical mastitis). Because different methods were used to contrast distributions, crude proportions were presented instead of the estimated ones after confirming that the observed differences in the models were in the same direction as the crude ones. All obtained *P*-values were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) method using the *p.adjust* function in R (Crawley, 2013).

NAS Species Prevalence. The objective of this analysis was to estimate and compare the NAS, *S. aureus*, *Strep. dysgalactiae*, *S. uberis*, and *Klebsiella* spp. prevalence in the low and high SCC categories. Using traditional frequentist statistical approaches, the prevalence of different bacteria could be severely underestimated according to the sensitivity and specificity of our definition of IMI. For example, for the NAS estimation according to the proposed definition of IMI, 6,302 NAS infections were identified. For unknown reasons, 721 isolates were not stored, leaving 5,581 NAS isolates for identification (0.88 of total NAS samples). From the 5,581 NAS isolates, 74 isolates were identified as another bacterial genus resulting in 5,507 NAS isolates. Still, according to Dohoo et al. (2009), the estimated sensitivity of using a single sample culture to diagnose an IMI (as done in our study) caused by a NAS is 24.2% and specificity is 100%. In this scenario, Bayesian analysis using the latent class approach can be used for prevalence estimation using the appropriate priors that would properly deal with the low sensitivities observed in our IMI definition for the different bacteria and for incomplete preservation of isolates meeting the preservation conditions. The latent class model used (illustrated in Figure 1) was similar to the one proposed by McInturff et al. (2004) and was as follows:

$$Y_{\text{collection}} \sim \text{Bernoulli}(P_{\text{collection}}),$$

$$P_{\text{collection}} = P_{\text{observed}} \times Se_{\text{collection}} + (1 - P_{\text{observed}}) \times (1 - Sp_{\text{collection}}) \text{ (Measurement error part 1),}$$

$$P_{\text{observed}} = P_{\text{population}} \times Se_{\text{IMI def}} + (1 - P_{\text{population}}) \times (1 - Sp_{\text{IMI def}}) \text{ (Measurement error part 2),}$$

$$\text{Logit}(P_{\text{population}}) = \beta_0 + \beta_1 x \text{ (Response part),}$$

where $Y_{\text{collection}}$ is the NAS species-specific status of an isolate available in the MPCC (e.g., is it or not a *S. chromogenes*) and is assumed to follow a Bernoulli distribution with a certain probability ($P_{\text{collection}}$) of having $Y_{\text{collection}} = 1$. This probability of observing a given NAS species in the MPCC can be linked to the probability of observing such an IMI among all samples collected

during this observational cohort study and identified as NAS IMI with the chosen IMI definition (P_{observed}) using the first measurement error part of the model. Thus, this part of the model represents the probability of an isolate that met inclusion criteria of actually being included in the MPCC (and the other way around). These probabilities can be linked using prior knowledge on probability for an isolate of a given species being included in the MPCC ($Se_{\text{collection}}$) and probability of an irrelevant isolate being wrongly included in the MPCC ($Sp_{\text{collection}}$). Then the probability of a sample being observed as positive to a given NAS with the used IMI definition (P_{observed}) can be linked to the true population prevalence of that NAS species ($P_{\text{population}}$) using the second measurement error part of the model. This can be achieved by including prior knowledge on sensitivity and specificity of the IMI definition chosen ($Se_{\text{IMI def}}$ and $Sp_{\text{IMI def}}$, respectively). The response part of the model can then be used to model the true prevalence of a given NAS species in the population as a function of different predictors, and while including any random intercepts, random slopes, and so on, as needed.

Different prior distributions were used and specificity estimates >99% were considered as 100% (Table 1). Distributions were truncated for values within 5 percentage points of the estimated sensitivities and specificities. For the analysis, misclassification was considered independent of SCC status. Misclassification of NAS species included 3 distinct priors: sensitivity for using a single sample to define IMI [β (66.97, 211.80)], sensitivity for samples that were not obtained/stored [β (136.5, 17.8)], and specificity for samples that were misclassified by our laboratory [β (56.99, 1.5)]; Figure

1. These values were used to adjust all NAS prevalence estimates. Misclassification of NAS species was assumed to be independent from the fact that a sample was stored or not, and also assumed to be common for all NAS species.

Initial analyses considered the complete structure of our data set (repeated measures clustered inside quarter, cow, and herd), including random slopes of DIM at the quarter level (to address autocorrelation). Random slopes were dropped in final models due to difficulties in properly estimating variance and covariance parameters. Final models for species commonly isolated contained the random effects of quarter, cow, and herd. The SCC categories were treated as fixed effects in individual models. Logit values obtained were transformed to population-averaged values (Dohoo et al., 2009) and prevalence estimates were obtained using the invert logit function in the associated coefficient using JAGS (Plummer, 2003). For rare outcomes (NAS species rarely isolated), models were built assuming independence of the observations. A Markov chain Monte Carlo method using Gibbs sampling was applied to estimate the posterior densities using the *runjags* package in R (Denwood and Plummer, 2016). Four chains running in parallel were part of 100,000 iterations in total for each pathogen. Plots of posterior distribution were visualized in R, and differences in prevalence estimates between SCC categories were considered significant when its respective 95% credible interval did not include zero.

Association Between NAS Species and SCC. Comparison of SCC between species was performed in R. First, SCC was natural log-transformed (**LnSCC**)

Figure 1. Estimates of sensitivity and specificity for non-*aureus* staphylococci (NAS) according to the structure of the data set. $Se_{\text{collection}}$ = probability for an isolate of a given species being included in the Mastitis Pathogen Culture Collection (MPCC); $Sp_{\text{collection}}$ = probability of an irrelevant isolate being wrongly included in the MPCC; $Se_{\text{IMI def}}$ = sensitivity of the IMI definition chosen according to Dohoo et al. (2009); $Sp_{\text{IMI def}}$ = specificity of the IMI definition chosen according to Dohoo et al. (2009).

to approximate the normal distribution. Visual presentation of the SCC distribution for each species was done using crude SCC data. Next, the association between DIM and SCC was studied using mixed-effects linear regression and scatter plots. Models with observations nested in lactation, quarter, cow, and herd were evaluated with DIM alone, and with its quadratic and cubic terms, respectively. The P -values obtained from these models and also plots of residual and predicted values suggested the utilization of all 3 polynomial terms, resulting in a model with 2 points of inflexion in the curve of $\text{LnSCC} \times \text{DIM}$ (beginning and middle/end of lactation for NAS combined). Multilevel mixed-effects linear regression was used to estimate LnSCC according to different bacterial species. Culture-negative quarters were used as a baseline value. Different random structures that dealt with the presence of autocorrelation in SCC measures over time were tested and compared based on the likelihood statistic and residual plots. A 5-level random-intercept model (herd, cow, quarter, lactation, and observation level) with random slopes of DIM, DIM squared, and DIM cubed at lactation and quarter levels fitted with maximum-likelihood resulted in the best fit, with successful estimation of all variance and covariance terms between intercept and slopes. Geographical region was included as a fixed effect to deal with the clustering effect at this level. Different NAS species were modeled as fixed effects, with negative quarters included as baseline. Parity and average milk production at the herd level were included as possible contextual confounders/effect modifiers. Interaction effects of DIM indicators and different NAS species were evaluated with 2nd order interaction terms between each bacteria indicator and DIM terms to study different shapes in the curvature of $\text{LnSCC} \times \text{DIM}$ according to the bacteria. The final model selected is described as

$$\begin{aligned} \text{LnSCC}_{ijklm} = & \beta_0 \text{cons} + \sum_{n=1}^{23} \beta_n \text{bacteria}_{ijklm} + \beta_{24} \text{DIM}_{ijklm} \\ & + \beta_{25} \text{DIM}_{ijklm}^2 + \beta_{26} \text{DIM}_{ijklm}^3 + \beta_{27} \text{heifer}_{ijklm} + \beta_{28} \text{avr_mp}_m \\ & + \varepsilon_{ijklm} \end{aligned}$$

with

$$\beta_0 = \beta_{0jklm} + \omega_{0m} + \theta_{0lm} + \vartheta_{0klm} + \mu_{0jklm},$$

$$\beta_{24} = \beta_{24jk} + \vartheta_{1klm} + \mu_{1jklm},$$

$$\beta_{25} = \beta_{25jk} + \vartheta_{2klm} + \mu_{2jklm},$$

$$\beta_{26} = \beta_{26jk} + \vartheta_{3klm} + \mu_{3jklm},$$

where i is the i th observation collected from the j th lactation from the k th quarter from the l th cow from the m th herd. LnSCC is the log-transformed SCC, β_{1-26} are the indicators for each bacteria. β_{27} is the indicator for whether the sample is from a primiparous or from an older cow, and β_{28} refers to the estimated rate of change in the LogSCC due to a unit increase in the herd average milk production of a cow (kg); both terms assume that the effects on the outcome (LogSCC) are common with respect to the other terms in the model and their effects are conditional on the components of the random structure. DIM, DIM², and DIM³ are the linear, quadratic, and cubic measured components of DIM; heifer is whether the observation comes from a heifer or a multiparous cow; avr_mp stands for average milk production at herd level in kilograms; and ε is the residual term. β_0 cons (or baseline value) is the estimated mean LogSCC for an average lactation from an

Table 1. Mean and lower and upper limits of prior distributions used in the present study according to various estimates of sensitivity (Se) and specificity (Sp) for several udder pathogens (or pathogen group)

Pathogen ¹	Parameter ²	Distribution	Mean	Limit	
				Lower	Upper
NAS culture 1	Se _{IMI def}	$\beta(66.97, 211.80)$	0.24	0.19	0.29
NAS culture 2	Se _{collection}	$\beta(136.5, 17.8)$	0.88	0.83	0.93
	Sp _{collection}	$\beta(56.99, 1.5)$	0.98	0.93	0.99
<i>Staphylococcus aureus</i>	Se _{IMI def}	$\beta(225.79, 101.87)$	0.69	0.64	0.74
<i>Streptococcus dysgalactiae</i>	Se _{IMI def}	$\beta(219.36, 80.12)$	0.73	0.68	0.78
<i>Streptococcus uberis</i>	Se _{IMI def}	$\beta(219.36, 80.12)$	0.73	0.68	0.78
<i>Klebsiella</i> spp.	Se _{IMI def}	$\beta(152.55, 218.13)$	0.41	0.36	0.46

¹NAS = non-*aureus* staphylococci; NAS culture 1 = NAS isolated from IMI as defined by the Canadian Bovine Mastitis Research Network (CBMRN); NAS culture 2 = NAS isolates received from CBMRN and finally identified as NAS species.

²Se_{collection} = probability for an isolate of a given species being included in the Mastitis Pathogen Culture Collection (MPCC); Sp_{collection} = probability of an irrelevant isolate being wrongly included in the MPCC; Se_{IMI def} = sensitivity of the IMI definition chosen according to Dohoo et al. (2009).

average quarter, cow, and herd, for a cow with 120 DIM in a herd where the average milk production is 32 kg per cow (centered values; ω stands for the m th herd-specific random effects components for the baseline value; θ are the k th cow-specific random effects components for the baseline value; whereas ϑ_0 stands for the baseline random effects specific for the k th quarter and j th lactation, μ_0 for the quarter- and lactation-specific effects on the linear (ϑ_1, μ_1), quadratic (ϑ_2, μ_2), and cubic (ϑ_3, μ_3) DIM terms of the model. Covariance between the different variance components was estimated but not presented in this model.

The same model was fitted using NAS as a group with other major bacteria. Estimates of the LnSCC for NAS as a group were also obtained. Contrasts of LnSCC values were then used for comparison between species, with Sidak's adjustment for multiple comparisons (Sidak, 1967). Those comparisons assumed that the differences between LnSCC for species were common for all parities, provinces, DIM, and average milk production, and were based on marginal estimates for

each of those factors. Comparison of LnSCC of NAS as a group versus different NAS species was performed using t -tests using the estimates of the different models and assuming inequality of variances. The P -values were adjusted for multiple comparisons using the $p.adjust$ function in R. Average LnSCC was converted to geometric mean SCC for presentation purposes.

RESULTS

Distribution of NAS Species

The 5,507 NAS isolates were obtained from 3,015 lactations of 2,859 quarters of 1,940 cows, of which 3,561 were from quarters with low SCC, 1,873 from quarters with high SCC, and 73 from clinical mastitis quarters (Table 2).

The 5 most common NAS species were commonly observed in all 3 categories, low SCC, high SCC, and clinical mastitis (Table 2). However, whereas the *S. chromogenes*, *S. simulans*, *S. epidermidis*, and *S. hae-*

Table 2. Distribution of non-aureus staphylococci (NAS) species within NAS as a group, isolated from bovine milk from 91 Canadian dairy herds between 2007 and 2008 from udder quarters with low (<200,000 cells/mL) and high ($\geq$ 200,000 cells/mL) SCC, and clinical mastitis

Staphylococcus species	Total		Low SCC		High SCC		Clinical mastitis	
	N	%	N	%	N	%	N	%
<i>S. chromogenes</i> ¹	2,683	48.72	1,794	50.38	866	46.24	23	31.5
<i>S. simulans</i> ¹	931	16.91	553	15.53	360	19.22	18	24.7
<i>S. xylosus</i> ¹	630	11.44	450	12.64 ²	178	9.50	2	2.7
<i>S. haemolyticus</i> ¹	433	7.86	259	7.27	169	9.02	5	6.9
<i>S. epidermidis</i> ³	231	4.19	117	3.29	108	5.77	6	8.2
<i>S. cohnii</i> ¹	140	2.54	109	3.06 ⁴	30	1.60	1	1.4
<i>S. sciuri</i> ³	131	2.38	92	2.58 ⁵	29	1.55 ⁵	10	13.7
<i>S. gallinarum</i> ¹	51	0.93	20	0.56 ⁴	30	1.60	1	1.4
<i>S. capitis</i> ¹	46	0.84	23	0.65	22	1.17	1	1.4
<i>S. arlettae</i> ⁶	46	0.84	29	0.81	15	0.80	2	2.7
<i>S. warneri</i> ⁶	31	0.56	17	0.48	14	0.75	—	0
<i>S. saprophyticus</i> ⁶	30	0.54	20	0.56	10	0.53	—	0
<i>S. agnetis</i> ⁶	26	0.47	5	0.14 ²	19	1.01 ⁷	2	2.7
<i>S. equorum</i> ⁶	24	0.44	22	0.62 ⁴	2	0.11	—	0
<i>S. succinus</i> ⁶	17	0.31	14	0.39	3	0.16	—	0
<i>S. hominis</i> ⁶	12	0.22	8	0.22	4	0.21	—	0
<i>S. devriesei</i> ⁶	10	0.18	5	0.14	4	0.21	1	1.4
<i>S. pasteurii</i> ⁶	8	0.15	5	0.14	3	0.16	—	0
<i>S. nepalensis</i> ⁶	7	0.13	7	0.20	—	0	—	0
<i>S. vitulinus</i> ⁶	6	0.11	4	0.11	2	0.11	—	0
<i>S. auricularis</i> ⁶	4	0.07	3	0.08	1	0.05	—	0
<i>S. hyicus</i> ⁶	4	0.07	1	0.03	3	0.16	—	0
<i>S. caprae</i> ⁶	3	0.05	1	0.03	1	0.05	1	1.4
<i>S. fleuretti</i> ⁶	2	0.04	2	0.06	—	0	—	0
<i>S. kloosii</i> ⁶	1	0.02	1	0.03	—	0	—	0
Total NAS	5,507	100	3,561	100	1,873	100	73	100

¹Significance determined by mixed linear modeling considering all cluster levels.

²Significantly ($P < 0.05$) different from high SCC.

³Significance among categories of IMI status determined by mixed linear model without accounting for clustering at quarter level.

⁴Tendency for difference ($0.05 < P < 0.10$) from high SCC.

⁵Significantly ($P < 0.05$) different from clinical mastitis.

⁶Significance determined by Fisher's exact test due to low number of subjects in each IMI status.

⁷Tendency for difference ($0.05 < P < 0.10$) from clinical mastitis.

molyticus positive quarters were evenly distributed throughout the 3 categories, *S. xylosus*, *S. cohnii*, and *Staphylococcus equorum* tended to be more frequently isolated from low SCC NAS-positive quarters. In contrast, among the NAS-positive quarters, *S. agnetis* (1%) and *S. gallinarum* tended to be more frequently isolated from high SCC quarters. *Staphylococcus sciuri* was more frequently and *S. agnetis* tended to be more frequently isolated from NAS-positive quarters with clinical mastitis than from NAS-positive quarters with low or high SCC.

Prevalence of NAS Species

Overall, NAS IMI was more prevalent in high than in low SCC quarters (50.6 and 20.6%, respectively) (Table 3). Quarters with a low SCC were more often culture-negative than high SCC quarters, whereas the opposite was the case for major udder pathogen-

positive quarters. The 11 most common NAS species were more prevalent in high SCC quarters. True *S. chromogenes* IMI prevalence was estimated at 8.7% in low SCC quarters and at 17.7% in high SCC quarters. For all other NAS, true IMI prevalence was <4% in low SCC quarters and <8% in high SCC quarters. Nevertheless, many NAS species (*S. simulans*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis*) had higher true IMI prevalence in high SCC quarters than major pathogens such as *Strep. dysgalactiae*, *Strep. uberis*, and *Klebsiella* spp. No NAS species was more prevalent in low than in high SCC quarters (Table 3).

SCC

Geometric mean SCC of NAS-positive quarters was approximately 70,000 cells/mL, which was higher than culture-negative quarters, and lower than quarters positive for major pathogens (Table 4; Figures 2 and

Table 3. True population prevalence of non-*aureus* staphylococci (NAS) species, major pathogens, and culture-negative quarters in quarters with a low (<200,000 cells/mL) and high (≥200,000 cells/mL) SCC in 91 Canadian dairy herds in 2007 to 2008¹

Species	Low SCC				High SCC			
	N	Prevalence	95% CrI		N	Prevalence	95% CrI	
<i>Staphylococcus chromogenes</i> ²	1,794	8.66 ^a	7.96	9.50	866	17.69	16.18	19.52
<i>Staphylococcus simulans</i> ²	553	3.50 ^a	3.10	4.01	360	7.87	6.93	8.97
<i>Staphylococcus xylosus</i> ²	450	3.58 ^a	3.04	4.16	178	5.90	4.79	7.00
<i>Staphylococcus haemolyticus</i> ³	259	1.52 ^a	1.22	1.91	169	4.65	3.67	5.92
<i>Staphylococcus epidermidis</i> ²	117	1.07 ^a	0.80	1.38	108	3.47	2.81	4.31
<i>Staphylococcus cohnii</i> ²	109	1.28	0.99	1.62	30	1.51	0.86	2.18
<i>Staphylococcus sciuri</i> ²	92	0.97 ^a	0.72	1.29	29	1.46	1.02	2.02
<i>Staphylococcus gallinarum</i> ²	20	0.34 ^a	0.17	0.53	30	1.20	0.82	1.68
<i>Staphylococcus capitis</i> ³	23	0.13 ^a	0.083	0.21	22	0.61	0.37	0.93
<i>Staphylococcus arlettae</i> ³	29	0.16 ^a	0.11	0.25	15	0.42	0.23	0.69
<i>Staphylococcus warneri</i> ³	17	0.10 ^a	0.06	0.16	14	0.38	0.21	0.65
<i>Staphylococcus saprophyticus</i> ³	20	0.12	0.07	0.18	10	0.28	0.13	0.51
<i>Staphylococcus agnetis</i> ³	5	0.03 ^a	0.01	0.06	19	0.52	0.31	0.83
<i>Staphylococcus equorum</i> ³	22	0.12	0.08	0.20	2	0.05	0.009	0.18
<i>Staphylococcus succinus</i> ³	14	0.08	0.04	0.14	3	0.08	0.02	0.22
<i>Staphylococcus hominis</i> ³	8	0.05	0.020	0.09	4	0.11	0.03	0.26
<i>Staphylococcus devriesei</i> ³	5	0.03	0.09	0.06	4	0.11	0.03	0.27
<i>Staphylococcus pasteurii</i> ³	5	0.03	0.01	0.06	3	0.08	0.02	0.22
<i>Staphylococcus nepalensis</i> ³	7	0.04	0.02	0.08	—	0.003	0	0.08
<i>Staphylococcus vitulinus</i> ³	4	0.02	0.006	0.06	2	0.05	0.007	0.18
<i>Staphylococcus auricularis</i> ³	3	0.02	0.004	0.04	1	0.03	0.001	0.14
<i>Staphylococcus hyicus</i> ³	1	0.005	0	0.02	3	0.08	0.02	0.22
<i>Staphylococcus caprae</i> ³	1	0.006	0	0.03	1	0.03	0	0.13
<i>Staphylococcus fleuretti</i> ³	2	0.011	0.001	0.04	—	0.003	0	0.08
<i>Staphylococcus kloosii</i> ³	1	0.005	0	0.03	—	0.004	0	0.08
Total NAS ²	3,561	20.60 ^a	17.80	25.46	1,873	50.60	43.83	62.09
Culture-negative ²	42,727	50.89 ^a	50.23	51.51	4,729	28.01	27.12	28.83
<i>Staphylococcus aureus</i> ²	434	1.82 ^a	1.61	2.05	1,642	10.78	9.94	11.71
<i>Streptococcus dysgalactiae</i> ²	21	0.27 ^a	0.18	0.37	164	1.83	1.54	2.16
<i>Streptococcus uberis</i> ²	48	0.41 ^a	0.32	0.54	182	1.83	1.57	2.17
<i>Klebsiella</i> spp. ²	22	0.33 ^a	0.22	0.49	47	1.16	0.88	1.51

^aCredible interval differences do not cross zero.

¹True prevalence was estimated using a Bayesian latent class model accounting for use of imperfect definition of intramammary infection and incomplete preservation of pathogen in a national mastitis pathogen culture collection. 95% CrI = Bayesian 95% credible interval.

²Prevalence was estimated considering the clustered nature of the data.

³Prevalence was estimated ignoring the clustered nature of the data.

Table 4. Somatic cell count of quarters culture-positive for non-*aureus* staphylococci (NAS) species, major pathogens, and culture-negative quarters from 91 dairy herds in 4 regions of Canada using mixed effects linear regression

Item	SCC $\times 10^3$ cells/mL		
	Geometric mean	95% CI	
		Lower	Upper
<i>Staphylococcus chromogenes</i>	67.86 ^{a,d}	63.05	73.03
<i>Staphylococcus simulans</i>	77.39 ^{a,c}	69.70	85.94
<i>Staphylococcus xylosus</i>	66.84 ^{a,d}	59.76	74.76
<i>Staphylococcus haemolyticus</i>	65.26 ^{a,d}	57.35	74.25
<i>Staphylococcus epidermidis</i>	66.99 ^{a,d}	56.56	79.35
<i>Staphylococcus cohnii</i>	46.27 ^{a,b,d}	37.84	56.57
<i>Staphylococcus sciuri</i>	54.52 ^{a,d}	44.10	67.40
<i>Staphylococcus gallinarum</i>	96.62 ^{a,c}	68.10	137.07
<i>Staphylococcus capitis</i>	123.36 ^{a,c}	84.43	180.24
<i>Staphylococcus arlettae</i>	63.58 ^{a,d}	45.34	89.15
<i>Staphylococcus warneri</i>	63.27 ^{a,d}	42.01	95.28
<i>Staphylococcus saprophyticus</i>	65.55 ^{a,d}	42.89	100.18
<i>Staphylococcus agnetis</i>	81.17 ^{a,c}	50.79	129.72
<i>Staphylococcus equorum</i>	40.80 ^{a,b,d}	25.38	65.57
<i>Staphylococcus succinus</i>	42.84 ^{a,b,d}	25.38	72.31
<i>Staphylococcus hominis</i>	29.40 ^{b,d}	14.88	58.07
<i>Staphylococcus hyicus</i>	85.65 ^{a,c}	68.92	106.45
Other NAS species ¹	47.52 ^{a,b,d}	33.12	68.19
Overall NAS	70.36 ^{a,d}	67.36	74.30
Culture-negative	32.29 ^{b,d}	30.63	34.03
<i>Staphylococcus aureus</i>	173.82 ^{a,c}	160.09	188.73
<i>Streptococcus dysgalactiae</i>	183.45 ^{a,c}	110.46	151.71
<i>Streptococcus uberis</i>	161.57 ^{a,c}	134.43	194.20
<i>Klebsiella</i> spp.	132.85 ^{a,c}	98.75	178.72

^aSignificantly ($P < 0.05$) higher than average SCC of negative-culture quarters.

^bSignificantly ($P < 0.05$) lower than NAS average SCC.

^cSignificantly ($P < 0.05$) higher than NAS average SCC.

^dSignificantly ($P < 0.05$) lower than major pathogens (*S. aureus*, *Strep. dysgalactiae*, *Strep. uberis*, and *Klebsiella* spp.) average SCC.

¹NAS species isolated in lower numbers (<10 isolates per species): *Staphylococcus devriesei*, *Staphylococcus pasteurii*, *Staphylococcus nepalensis*, *Staphylococcus vitulinus*, *Staphylococcus auricularis*, *Staphylococcus caprae*, *Staphylococcus fleuretti*, and *Staphylococcus kloosii*.

3). Average SCC of NAS as a group was mostly due to 4 of the 5 most common species, *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis* (Table 4; Figures 4 and 5). The SCC distribution of quarters positive for these 4 species is similar (Figures 4 and 5). Among the NAS species, *S. simulans*, *S. gallinarum*, *S. capitis*, *S. agnetis*, and *S. hyicus*-positive quarters had a higher geometric mean SCC than NAS as a group (Table 4). The SCC distribution of quarters positive for *S. simulans*, *S. gallinarum*, and *S. capitis* was more skewed toward higher SCC values than *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis* (Figures 4 to 6). In contrast, *S. cohnii*, *S. equorum*, *S. succinus*, *S. hominis*, and other less frequent NAS species had a SCC that was lower than NAS as a group (Table 4). The SCC distribution of quarters positive for *S. sciuri* and *S. cohnii* was more skewed toward lower SCC values than *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis* (Figures 4 to 6). Samples positive for most NAS species had, on average, an SCC lower

than quarters positive for the major pathogens *S. aureus*, *Strep. dysgalactiae*, *Strep. uberis*, and *Klebsiella* spp. (Table 4).

DISCUSSION

In this large cohort study, prevalence of IMI with almost all NAS species was higher in the high SCC than in low SCC quarters (Table 3). However, the distribution of NAS species was different in high SCC quarters from low SCC quarters. Additionally, different NAS species had diverse effects on SCC, as has been previously reported in smaller studies for several NAS species earlier (Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2016).

Prevalence of NAS IMI at the quarter level has been reported to be as low as ~11% (Gillespie et al., 2009; Sampimon et al., 2009a) and as high as 25% (Supré et al., 2011). These variations included a wide range of sample selection criteria, sampling methods, laboratory

Figure 2. Distribution of SCC of culture-negative quarters and non-*aureus* staphylococci (NAS) and *Staphylococcus aureus*-positive quarters.

procedures, and IMI definitions, as well as, intrinsic variation for each herd and region under investigation and its respective management practices, all of which could have contributed to differences in results (Barke-
ma et al., 2006; Compton et al., 2007; Sampimon et al., 2009a). Recent literature has demonstrated the importance of molecular identification at the species level as the first step toward the investigation of species-specific effects of NAS on udder health (Supré et al., 2011;

Fry et al., 2014; Tomazi et al., 2015). Methods used in previous research, particularly phenotypic methods, have resulted in species misidentification. For example, Sampimon et al. (2009a) reported that *S. haemolyticus* was one of the most problematic species with regard to specificity of species identification methods; partially discrepant results in reported average SCC might have resulted from this problem. A similar problem could occur at the molecular level. Because *S. agnetis*, *S. hyicus*

Figure 3. Distribution of SCC of non-*aureus* staphylococci (NAS), *Streptococcus dysgalactiae* and *uberis* (combined), and *Klebsiella* spp.-positive quarters.

Figure 4. Distribution of SCC of quarters positive for the 3 most frequently isolated non-aureus staphylococci species (*Staphylococcus chromogenes*, *Staphylococcus simulans*, and *Staphylococcus xylosus*).

and *S. chromogenes* are phylogenetically very similar, the older reports on the effect of *S. hyicus* on SCC could have been overestimated due to misidentification, whereas effects of *S. agnetis* are likely under-recognized (Taponen et al., 2012).

Staphylococcus chromogenes, *S. simulans*, *S. haemolyticus*, and *S. epidermidis* were evenly distributed across SCC levels, although the prevalence range was different from Thorberg et al. (2009) and Persson Waller et al. (2011). Supré et al. (2011) reported the same most common species. Furthermore, other NAS species such as *S. arlettae*, *S. devriesei*, *S. equorum*, *S.*

fleuretti, *S. hyicus*, *S. kloosii*, *S. pasteurii*, and *S. warneri* were isolated in equal proportions in low SCC and high SCC quarters in the present study, as was also described by Thorberg et al. (2009), Persson Waller et al. (2011), and Supré et al. (2011).

Taponen et al. (2006) reported that *S. haemolyticus* occurred most frequently in subclinical mastitis cases, which coincides with its increased prevalence in high SCC quarters in the present study. It is important to note that *S. haemolyticus* had an equal proportional distribution in all nonclinical and clinical mastitis samples. The same discrepancies among distribution and

Figure 5. Distribution of SCC of quarters positive for the 4th to 6th most frequently isolated non-aureus staphylococci species (*Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, and *Staphylococcus cohnii*).

Figure 6. Distribution of SCC of quarters positive for the 7th to 9th most frequently isolated non-*aureus* staphylococci species (*Staphylococcus sciuri*, *Staphylococcus gallinarum*, and *Staphylococcus capitis*).

prevalence in our study were observed with *S. xylosus* and *S. sciuri*, which do not increase SCC according to most experimental studies (Sampimon et al., 2009a; Thorberg et al., 2009). However, according to Supré et al. (2011), and the prevalence estimate reported herein, *S. xylosus* is also a relevant species in high SCC cases. In contrast, Thorberg et al. (2009) described *S. sciuri* only in cases without an elevation in SCC.

Although among NAS-positive samples *Staphylococcus capitis*, *S. arlettae*, and *S. warneri* had a similar distribution in low and high SCC samples, they were more prevalent in high SCC samples, indicating that these species were associated with a similar increase in SCC as the NAS as a group. In contrast, *S. gallinarum*, *S. agnetis*, *S. capitis*, *S. agnetis*, and *S. hyicus* not only had a higher prevalence in high SCC quarters, but also were among the NAS overrepresented in high SCC quarters (Table 3), and had a higher average SCC than NAS as a group (Table 4). Based on these differences in SCC, we inferred that although NAS as a group is associated with a higher SCC, some species such as *S. gallinarum* and *S. agnetis* increased SCC more and can therefore be considered relatively more pathogenic.

Worldwide, the prevalence of NAS as a group in clinical mastitis quarter cases has been reported as 5.4% (Steenefeld et al., 2008), 6 to 7.5% (Olde Riekerink et al., 2008; Persson Waller et al., 2009; Piessens et al., 2011; Oliveira et al., 2013), 9 to 10.8% (Oliver and Jayarao, 1997; de Haas et al., 2002), and 20% (Levison et al., 2016) of cases. Among NAS species, *S. chromogenes* was the most common species isolated from

cases of clinical mastitis in the present study, similar to that reported by Zadoks and Watts (2009). In contrast, other researchers have reported *S. simulans* to be the most frequently isolated NAS species in clinical mastitis (Myllys, 1995; Waage et al., 1999; Taponen et al., 2008; Thorberg et al., 2009). Once again, reports vary regarding the importance of *S. xylosus* and *S. haemolyticus* in clinical mastitis. According to Persson Waller et al. (2011), *S. xylosus* and *S. haemolyticus* were isolated from clinical mastitis cases at the same frequency as from subclinical mastitis, whereas Taponen et al. (2006) did not detect *S. xylosus* in clinical mastitis.

Other species, such as *S. equorum*, *S. warneri*, *S. hyicus*, and *S. sciuri*, have been reported as causes of clinical mastitis (Taponen et al., 2006; Piessens et al., 2011); however, in the present study the first 3 of these NAS species were not isolated from clinical mastitis cases. *Staphylococcus sciuri*, however, was isolated relatively frequently from clinical mastitis cases. This NAS species has been reported by other researchers to not increase SCC (Sampimon et al., 2009a; Thorberg et al., 2009), but has been observed in clinical mastitis (Taponen et al., 2006; Frey et al., 2013). Future work will examine whether *S. sciuri* has virulence factors that might influence its pathogenicity.

A total of 11 NAS species were associated with a higher SCC than the average SCC of culture-negative quarters (Table 4). The most frequently isolated NAS species, *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, and *S. epidermidis*, were associated with an increase in SCC (to 60,000 and 70,000 cells/mL), whereas the

highest SCC was found in quarters with a *S. agnetis*, *S. capitis*, *S. hyicus*, *S. gallinarum*, and *S. simulans* IMI. However, these levels were lower than SCC of the studied major pathogens. The newly described species, *S. agnetis* (Taponen et al., 2012), which is closely related to *S. hyicus* and *S. chromogenes*, was also associated with an increased SCC (Calcutt et al., 2014; Naushad et al., 2016; Condas et al., 2017).

Staphylococcus chromogenes, *S. epidermidis*, and *S. simulans* were reported as the NAS species that increase SCC most (Sampimon et al., 2009a; Taponen and Pyörälä, 2009; Thorberg et al., 2009; Persson Waller et al., 2011). According to Waage et al. (1999) and Taponen et al. (2006), *S. simulans* is the species with the most capacity to increase SCC, as it provokes the strongest inflammatory response based on experimental trials (Simojoki et al., 2011) and previous reports of naturally occurring IMI (Sampimon et al., 2009a; Thorberg et al., 2009; Supré et al., 2011). These findings concur with the present study; among the NAS species, SCC of quarters with a *S. simulans* IMI was only lower than those with *S. capitis* IMI. Thorberg et al. (2009) reported moderate to high increases in SCC by *S. epidermidis*, which has also been demonstrated experimentally (Simojoki et al., 2011), and was observed in relationship to culture-negative quarters in the current study. In the present study, the SCC of *S. epidermidis*-positive quarters was not particularly high; it was similar to *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, *S. arlettae*, *S. warneri*, and *S. saprophyticus*-positive quarters, and therefore to the SCC of NAS-positive quarters as a group (Table 4).

Staphylococcus chromogenes IMI was previously associated with a higher SCC and has been reported as an important species in quarters with a high SCC, persistent IMI, and clinical mastitis (Persson Waller et al., 2011; Supré et al., 2011; Fry et al., 2014). Simojoki et al. (2009) demonstrated that *S. chromogenes* strains were capable of increasing SCC and causing clinical mastitis. Furthermore, Fthenakis and Jones (1990) demonstrated intense neutrophil infiltration into mammary tissue of sheep after infection with different NAS species. Conversely, *S. chromogenes* could also be a protective species against IMI by major pathogens, as reported by Breyne et al. (2015). In that study, strains isolated from the teat apex caused less SCC increase compared with strains isolated from IMI, which might explain variations in the SCC and distribution of *S. chromogenes* in milk with different SCC levels also observed in the current study, leading also to a more moderate increase in SCC.

Contradictory findings were also reported for other NAS species. Chaffer et al. (1999), Fry et al. (2014), and Tomazi et al. (2015) reported that *S. haemolyticus*

had inflammatory potential in the udder. In contrast Thorberg et al. (2006), Sampimon et al. (2009a), and Supré et al. (2011) reported that *S. haemolyticus* was less inflammatory than some of the other NAS species. Other researchers have reported that *S. xylosus* increased SCC (Sampimon et al., 2009a; Supré et al., 2011; Fry et al., 2014), but similar to the present study, Thorberg et al. (2009) reported low CMT scores that were corroborated by a transient increase of SCC and no tissue abnormalities in another study (Fthenakis and Jones, 1990).

Species with lower than the average SCC for all NAS species were *S. cohnii*, *S. equorum*, *S. sciuri*, and *S. succinus*. In other studies, *S. cohnii*, *S. equorum*, *S. saprophyticus*, *S. sciuri*, *S. arlettae*, *S. succinus*, *S. pasteurii*, *S. auricularis*, *S. nepalensis*, *S. hominis*, and *S. gallinarum* did not increase quarter SCC in comparison to culture-negative samples (Thorberg et al., 2009; Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2016). Although our results are consistent with SCC averages for most NAS species, *S. gallinarum* was associated with an elevated average SCC. We previously demonstrated that the prevalence of IMI with this species is high immediately after calving (Condas et al., 2017). Perhaps the average SCC increases observed were due to higher SCC values in the early lactating period, thus overestimating its harmful effect on udder health. The same reasoning might be relevant for *S. capitis*, a species previously reported to affect SCC in 2 previous studies (Sampimon et al., 2009a; Fry et al., 2014).

CONCLUSIONS

It is well established that NAS is the most commonly isolated group of bacteria from bovine milk samples worldwide; therefore, understanding their effect on udder health is essential. Even though IMI with NAS increases SCC, the increase is relatively small, and the average SCC increase in the present study fell between culture-negative and major pathogen-infected quarters. Some species of NAS, particularly *S. sciuri*, are involved in clinical mastitis, indicating that they likely play a role as pathogens. The large data set available in this cohort allowed us to establish that *S. chromogenes*, *S. simulans*, and *S. sciuri* were the species most frequently isolated from clinical mastitis, whereas *S. agnetis*, *S. capitis*, *S. hyicus*, *S. gallinarum*, and *S. simulans* increased SCC the most. Identification of NAS species made it possible to determine how they affect udder health, and will direct further studies on virulence factors, host susceptibility, and management strategies focused on specific characteristics. Furthermore, future research may be focused on the investigation of possible species-specific NAS genotypes and how they

are related to aspects of mastitis, such as persistence, increased inflammatory response, decreased milk production, and if any genotypes confer protection against major pathogens.

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