***Interpretive summary***

The current study identifies which *Staphylococcus* and *Mammaliicoccus* (SaM) species are most relevant to udder health for organic dairies. It describes how quarter somatic cell count (SCC) varies as a result of intramammary infection with the most frequently isolated SaMspecies. Species-specific effect on quarter SCC for SaM has not been well-described for a population of certified organic dairies. Compared to healthy quarters, SCC was higher in quarters infected with 9 of 10 SaM identified. Although the increase in quarter SCC was modest for most SaM observed, their widespread nature can still result in an increased bulk tank SCC.

***Running head:***

Staphylococci mastitis on organic dairy farms

***Title***

Staphylococci and mammaliicocci: which species are important for udder health on organic dairy farms?

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***Abstract***

Variation in species distribution and diversity of staphylococci and mammaliicocci (SaM) causing intramammary infections in dairy cattle is associated with different management practices. Disparate selective pressures on organic dairies could potentially result in population differences of these mastitis-causing bacteria. The species-specific effect on quarter somatic cell count of SaM for a population of certified organic dairies has not been well-described. The current study presents data from a longitudinal, cross-sectional study of 10 certified organic dairy farms. The objective was to estimate how quarter somatic cell count (qSCC) varied as a result of infection with the most frequently isolated SaM species. Aerobic culture of quarter-milk samples to identify IMI was conducted in parallel with determination of qSCC. A linear hierarchical repeated measures mixed model was used to estimate qSCC for quarters with an IMI caused by a given SaM species, compared to healthy (no growth) quarters. The model included days in milk at time of sampling to adjust qSCC estimates for each SaM species. The final data set consisted of 648 quarters with an IMI due to 10 different SaMspp. and 1,972 healthy quarters. *S. chromogenes* was the most frequent species, followed by *S.* *aureus, S. haemolyticus,* and *S.* *simulans.* A large amount of variability was observed in the somatic cell score for healthy quarters and those infected with many SaM spp., especially *S. chromogenes, S. haemolyticus, S. simulans,* and *S. aureus.* Somatic cell score was significantly higher in quarters infected with *S. agnetis, S. aureus, S. chromogenes, S. devriesei, S. haemolyticus, S. hyicus, S. simulans, S. warneri, and S. xylosus* compared to healthy quarters. The highest cell count was for quarters infected with *S. warneri,* followed by *S.* *aureus, S. agnetis,* and *S. hyicus.* The relative distribution of various SaMspecies and their effect on qSCC in this population of small to midsize organic farms was similar to previous studies conducted on conventionally-managed dairies. Although the increase in qSCC was modest for most SaM species observed, the widespread nature of these intramammary pathogens can still result in sizeable increases in bulk tank SCC.

***Keywords:***

Mastitis, organic dairy cattle, staphylococci and mammaliicocci, quarter-level somatic cell count, intramammary infection

***Introduction***

Staphylococci and mammaliicocci are the predominant pathogens causing intramammary infections in dairy animals globally. Broadly, this group (herein abbreviated as SaM), includes the major mastitis pathogen *Staphylococcus aureus*, and a heterogeneous group of bacteria known as the non-*aureus* staphylococci and mammaliicocci. For many dairy farms that have implemented modern mastitis control practices minimizing the effects of “major” pathogens such as *S. aureus*, the leading contributor to bulk tank milk SCC on farms with good milk quality is IMI due to non-*aureus* staphylococci and mammaliicocci (NASM) (Schukken et al., 2009). Cow-level prevalence for NASM in one US study was 71% (Jenkins et al., 2019), and quarter-level prevalence of 11, 26, 21, and 33% has been reported in the US, Canada, and two Belgian studies, respectively (Condas et al., 2017a; Rowe et al., 2019; Wuytack et al., 2020; Valckenier et al., 2021). Although primarily associated with cases of subclinical mastitis (Persson Waller et al., 2011; Heikkilä et al., 2018), NASM are also capable of causing clinical mastitis (Taponen et al., 2007; Simojoki et al., 2009; Verbeke et al., 2014; Condas et al., 2017b; Wuytack et al., 2020). Taken as a group, IMI due NASMare reported to have minimal detrimental effect on milk yield (Tomazi et al., 2015; Valckenier et al., 2020) and can have a high rate of spontaneous cure (Taponen et al., 2007; Valckenier et al., 2020), but many NASM species have been shown to increase somatic cell count (Supré et al., 2011; Tomazi et al., 2015; Condas et al., 2017b; Valckenier et al., 2019), as well as persist for long periods of time in the udder (Piessens et al., 2011; Nyman et al., 2018; Valckenier et al., 2021).

NASMare an incredibly heterogenous group of bacteria, with studies identifying at least 25 different species as causing IMI in dairy cattle (Condas et al., 2017a; De Visscher et al., 2017). Different NASM species vary widely in both their epidemiology and ecology; some are considered primarily host-adapted (colonizing the skin or udder), while others are primarily found in the cow’s environment (as reviewed in De Buck et al., 2021). Certain species have been associated with stall surfaces, air, and unused sawdust bedding material (Piessens et al., 2011), some with different facility types (Condas et al., 2017a), and others with environmental contamination and poor teat hygiene at milking time (De Visscher et al., 2016; De Visscher et al., 2017). NASM also differ in how they behave as intramammary pathogens; the ability to cause persistent infections varies by species (Nyman et al., 2018; Valckenier et al., 2021), as well as the presence of antimicrobial resistance determinants (Frey et al., 2013; Fergestad et al., 2021), virulence potential (Naushad et al., 2019; França et al., 2021), and interaction with a host’s immune system (Åvall-Jääskeläinen et al., 2013; Breyne et al., 2015).

Perhaps most importantly for the overall udder health status of a dairy farm as measured by bulk tank SCC, NASM species also vary in the degree to which they cause an inflammatory reaction in the udder (Supré et al., 2011; Nyman et al., 2018; Wuytack et al., 2020; Taponen et al., 2022). However, a limited number of studies have described the effect of the breadth of observed species on quarter-level SCC using observations from multiple herds, where isolates were identified using MALDI-TOF or genotypic methods, and accounting for days in milk at time of observation (Fry et al., 2014; Condas et al., 2017b). Although infection status is the most important factor, stage of lactation has a significant effect on SCC (Schutz et al., 1990; Schepers et al., 1997). The relevance of different NASM species for udder health (as measured by species-specific effect on quarter SCC) is not well-described for certified organic dairy farms. Although similar in many herd management aspects, organic and conventional dairies differ in a number of ways including use of nutritional and veterinary support and vaccination (Stiglbauer et al., 2013), and treatments and attitudes around mastitis (Ruegg, 2009). For example, in the absence of antibiotic use on organic dairies, antimicrobial susceptibility of common mastitis pathogens can differ between conventional and organic dairy farms in the US (Tikofsky et al., 2003; Pol and Ruegg, 2007; Bombyk et al., 2008). Given that variation in NASM species distribution and diversity is associated with a variety of different management practices (Dufour et al., 2012; Condas et al., 2017a), it is possible that these differences may create disparate selective pressures between conventional and organic farms, potentially resulting in differences in bacterial virulence and impact on SCC.

The current study presents data from a longitudinal, cross-sectional study of 10 certified organic dairy farms in Vermont, US. Microbiological analyses of quarter-milk samples to identify IMI due to staphylococci and mammaliicocci were conducted in parallel with determination of quarter-level somatic cell count. The objective of this study was to estimate how quarter-milk SCC varied as a result of infection with the most frequently isolated SaM, in order to identify which species were more relevant to udder health in this population of farms.

***Materials and methods***

STROBE-VET (Strengthening the Reporting of Observational Studies in Epidemiology–Veterinary Extension) statement guidelines were followed in the reporting of this study (O'Connor et al., 2016). Animal use for this project was approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC; protocol #19-001).

*Sample origination*

Samples included in the current study were collected during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US) carried out in Winter 2019-2020. Enrolled farms were a non-probability subsample of certified organic dairies in Vermont which had participated in previous studies, and inclusion criteria included: 1) milking between 35-120 cows and 2) using either a tiestall barn bedded with shavings/sawdust or a deep bedded pack system to house lactating dairy cows. For the purposes of a separate study, an equal number of herds using each of the two bedding types were enrolled. Around the time of the first farm visit, herd records were captured from the record processing center working with 9 of the participating herds (Lancaster DHIA, Manheim, PA; Dairy One Co-Op. Inc., Ithaca, NY) to obtain freshening date and parity for the current lactation. Freshening date and parity for 1 herd was obtained from personal communication with the producer who kept written records. The goal was to enroll 35 cows of varying parity in early- to mid-lactation from each herd for the duration of the study. In 1 herd with approximately 35 lactating cows, all cows were sampled. In 8 herds with ≥ 35 cows and with available DHIA data, a stratified random approach was used with cows stratified by SCC, lactation number, and DIM and then randomly selected across these variables. In 1 herd with ≥ 35 cows and no DHIA data, the producer generated a list of 35 cows in early lactation so that they would continue to be milking for the duration of the study. Cows that were unable to be sampled at a follow-up visit (dried off, left the herd) were replaced with another lactating cow dictated by convenience. At each farm visit, duplicate quarter-milk samples were aseptically collected from each lactating quarter immediately before milking for all enrolled cows according to NMC guidelines (NMC, 2017). Briefly, after routine pre-milking teat disinfection was completed, researchers (wearing clean disposable gloves) scrubbed teat ends and the distal third of teats with 70% isopropyl alcohol-moistened gauze swabs until teat ends were visibly clean, stripped the quarters (discarding 3-5 squirts of foremilk), and sequentially collected approximately 5-6 mL of milk into each of two sterile 11-mL flip-top vials (Thermo Scientific CNLL500). Samples were kept on ice in a cooler during transport until stored temporarily overnight at 4°C in the laboratory, where an aliquot was frozen for SCC measurement and the remaining milk sample was processed for bacteriological culture.

*SCC measurement*

Aliquots of frozen quarter-milk samples were sent to the Vermont State Agricultural and Environmental Laboratory, where samples were gradually thawed under refrigeration at time of processing and quarter-level somatic cell count was determined using flow cytometry (Somacount FC, Bentley Instruments).

*Aerobic culture of milk samples and determination of bacteriological status*

Standard aerobic bacteriological culture of quarter-milk was performed in duplicate within 24 hours of collection to identify bacterial species present in the sample. After being homogenized by gentle inversion, tryptic soy agar plates with 5% sheep blood (Northeast Laboratory, Waterville, ME) were inoculated with 10 μL of milk using disposable calibrated plastic inoculating loops. Plates were then incubated in aerobic conditions at 37°C before being read at approximately 24 and 48 hrs.

Aerobic culture results of both samples were then used together to determine the overall bacteriological status of each quarter-milk sample into the following categories: 1) “no significant growth,” when there was no growth on both plates, or ≤ 200 CFU/mL on one plate and no growth on the other plate, or ≤ 200 CFU/mL on both plates and morphology of isolates on each plate was different; 2) “pure culture,” when there was ≥ 100 CFU/mL of a particular isolate identified with the same morphology on both plates; 3) “mixed culture,” when there was ≥ 100 CFU/mL of two phenotypically-distinct isolates identified, each growing on both plates; 4) “contaminated,” when 1 or both of the 2 samples had more than 2 morphologically distinct isolates growing on a plate; 5) and “indeterminate,” when the set of quarter-milk samples did not meet the criteria for any of the previous categories (e.g., missing duplicate). Quarter-day observations were included in this study when the bacteriological status of a quarter on a given day could be determined.

*Speciation of bacterial isolates*

Isolates from both pure and mixed culture quarter-milk samples were selected and grown in pure culture on blood agar. Standard benchtop tests were done to presumptively identify bacteria following NMC procedure guidelines, including differential growth on selective media, colony morphology, hemolytic pattern, catalase reaction, Gram stain, and coagulase testing (NMC, 2017) . Isolates were preserved in tryptic soy broth with a final concentration of 15% glycerol in cryovials and stored at -80°C. Frozen isolates were sent overnight on ice to the University of Missouri for speciation using MALDI-TOF mass spectrometry (Microflex, Bruker Daltonics) with Flex Control software (Bruker Daltonics). The protocol for identifying bacterial isolates with MALDI-TOF mass spectrometry has been described previously (Adkins et al., 2022). Briefly, generated spectra were assigned a score based on similarity with spectra in the manufacturer’s database (MBT 8468 MSP Library), as well as the University of Missouri laboratory custom database (Adkins et al., 2018). The confidence levels used for species identification were applied as previously described (Cameron et al., 2017), in which ≥ 1.7 was used for staphylococcal and mammaliicoccal species-level identification and < 1.7 was classified as inconclusive. Suspect staphylococci and mammaliicocci isolates unable to be identified to the species level and those identified as *Staphylococcus agnetis* or *Staphylococcus hyicus* by MALDI-TOF were speciated using *tuf*gene sequences with a cut-off of 98% identity as previously described (Hwang et al., 2011).

*Determination of IMI status and selection of data set*

Using the bacteriological status and speciation information, a quarter-day IMI status was assigned to each quarter observation: 1) “healthy,” when there was no significant growth; 2) “infected with a single SaM species,” when ≥ 100 CFU/mL of a particular SaM species was identified in pure culture on both plates (interpretation in series; Dohoo et al., 2011); 3) “infected with 2 SaM species,” when ≥ 100 CFU/mL of 2 different SaM species were identified in mixed culture on both plates; 4) “infected with non-SaM species,” when ≥ 100 CFU/mL of a non-SaM species was identified in pure or mixed culture on both plates (possibly in combination with a SaM species); and 5) “unknown” if the sample status had been identified as contaminated or indeterminate as previously described.

A quarter-day observation was included in the final data set if: 1) the IMI status was classified as healthy *or* infected with a single SaM species for any of the most frequently observed SaM species (≥ 5 observed IMI); 2) it was collected from a cow ≤ 305 DIM at time of observation; and 3) it had an associated quarter-level SCC measurement. Figure 1 depicts the selection of the final data set of quarter-day observations using these criteria.

*Statistical analysis*

The quarter-day somatic cell counts, quarter-day IMI status, cow parity and DIM data were organized into a spreadsheet (Microsoft Excel, Redmond, WA) and imported into the R Statistical Programming Environment (R Development Core Team, 2023) for analysis. Raw quarter-day-level SCC was converted to SCS [log2(quarter somatic cell count/1000) + 3] in order to address the non-normal distribution of SCC data. Descriptive statistics and visualizations were generated for the variables of interest (SCS, quarter-day IMI status, DIM) to evaluate the distribution and integrity of the data set and identify any missing values. Descriptive statistics and visualizations were also generated to describe the hierarchical structure of the data set (number of samples per quarter, number of quarters per cow, and number of cows per herd) to evaluate the distribution and integrity of the data and identify any missing values.

A linear hierarchical repeated measures mixed model was fitted to the data set in order to compare SCS of quarters infected with a single SaM species to healthy quarters. The “lme” function of the “nlme” package was used to build this model, in which the SCS of a quarter on a given day was the outcome variable, and the quarter-day IMI status (with healthy quarters as the reference value) was the main fixed predictor. Interaction between parity and quarter-day IMI status was evaluated to allow the effect of a given IMI to vary as function of age. Similarly, interaction between DIM (as a third degree polynomial variable) and quarter-day IMI status was evaluated to allow the effect of a given IMI to vary as function of DIM. Interaction terms were removed whenever the F-test for these terms yielded a *P*-value < 0.05. Finally, if the DIM by quarter-day IMI status interaction was not significant, then DIM was still kept as a fixed predictor in the model (again as a third degree polynomial variable), but not as part of an interaction, to allow it to adjust our SCS estimates as a function of DIM.

The hierarchical structure of the data was addressed by fitting random intercepts for quarter, cow, and herd (observations nested within quarter, quarters nested within cow, and cow within herd). Samples collected at different time points for a given quarter were considered repeated measurements, and a spatial exponential correlation structure was used to account for both the correlation between milk samples collected on the same quarter, and for the variation of this correlation with the varying amount of time between sample collections. The model (without interaction) was:

SCS*ijkl* = β0 + β1 Q-D-IMI status*ijkl* + β2DIM*ijkl* + β3DIM*ijkl*2 + β4DIM*ijkl*3 + v*l* + u*kl* + w*jkl* + e*ijkl*,

where SCS*ijkl* is the predicted SCS for the *i*th sample of the *j*th quarter of the *k*th cow from the *l*th herd; β0 is the intercept; β1, β2, β3, and β4 are the regression coefficients for quarter-day IMI status, and DIM as a third degree polynomial variable (to correct for the nonlinear relationship between DIM and SCS); and *vl*, *ukl*, *wjkl*, and *eijkl*are the herd random effect, cow random effect, quarter repeated effect, and sample error term, respectively (approximate normal distribution assumed). Statistical significance was determined using an F-test for interaction terms and a t-test for fixed effects, with significance declared at *P* ≤ 0.05. Final model fit was assessed by checking the homoscedasticity and normality of residuals (graphing of residuals vs. predicted values and Q-Q plots, respectively).

***Results***

Participating herds milked an average of 69.5 cows (median: 70; range: 44-105) of various breeds. Three visits were completed at 8 farms, 1 herd was sampled twice, and 1 was sampled 4 times before interruption by the COVID-19 pandemic. On average, 33.6 days elapsed between sequential farm visits for each herd (median: 34; range: 27-43). Five farms housed cows in a tiestall bedded with wood shavings, and 5 utilized a deep bedded pack system (3 actively managed for composting, 2 static).

The initial data set included 3,331 quarter-level observations, with 22 different species of staphylococci and mammaliicocci identified. SaM species causing IMI excluded from further analyses due to having < 5 IMI observations included: *M. fleurettii, M. sciuri, M. vitulinus, S. auricularis, S. capitis, S. cohnii, S. epidermidis, S. gallinarum, S. hominis, S. pseudintermedius, S. saprophyticus,* and *S. succinus*. The final data set consisted of 2,260 observations: 648 quarters with an IMI due to 10 different SaM (each causing at least 5 IMI), and 1,972 healthy quarters. Observations included in the final data set came from 1,272 quarters of 360 cows across all 10 herds included in the field study. The mean (median; range) number of cows included per herd was 36 (36; 34-39), whereas the number of quarters included per cow was 3.5 (2; 1-4). The mean number of observations per quarter included was 2.1 (2; 1-4). Twenty-seven percent of observations were the sole observation contributed to the data set by a given quarter, 41% came from quarters contributing 2 time points, and 31% and 1% came from quarters contributing 3 and 4 observations, respectively. The average time elapsed between sequential observations of a quarter was 37.1 days (median: 34.5; SD: 11.6), with an overall range of 27-96 days.

*S. chromogenes* was the most frequent species (59% of quarter observations with a SaMIMI), followed by *S. aureus* (17%)*, S. haemolyticus* (6%)*,* and *S. simulans* (5%)*.* A large amount of variability was observed in the SCS for healthy quarters and those infected with a number of different SaM species, especially *S. chromogenes,* *S.* *haemolyticus, S. simulans,* and *S. aureus* (observed quarter SCS data presented in Figure 2). The observed SCS for *S. chromogenes* IMI ranged from -2.6 to 8.9 (median: 3.3; equivalent to 2,000 cells/mL to 6.1 million cells/mL), with 29.7% of observations having a SCS ≥ 4.0. The observed SCS for *S. aureus* IMI ranged from 0.6 to 10.5 (median: 5.9; equivalent to 8,000 cells/mL to 18 million cells/mL), with 87.5% of observations of having an SCS ≥ 4.0. The observed SCS for *S. haemolyticus* IMI ranged from -2.1 to 6.1 (median: 3.5; equivalent to 3,000 cells/mL to 880,000 cells/mL), with 33.3% of observations having a SCS ≥ 4.0. The observed SCS for *S. simulans* IMI ranged from -0.8 to 6.7 (median: 3.4; equivalent to 7,000 cells/mL to 1.3 million cells/mL), with 37.1% of observations having a SCS ≥ 4.0.

In a model comparing SCS of quarters infected with SaM to healthy quarters and adjusted for DIM with an interaction term between IMI status and parity, the interaction between IMI status and parity was not significant (*P* = 0.86); thus, effect of the quarter IMI status on SCS was the same, regardless of parity for this data set. In a model comparing SCS of quarters infected with SaM to healthy quarters and adjusted for DIM with an interaction term between IMI status and DIM, the interaction between IMI status and DIM was not significant (*P* = 0.25). This meant that both IMI status and DIM affected SCS, but that the effect of IMI status on SCS did not vary as function of DIM for these data. We could, therefore, remove the interaction with DIM. The final model results comparing SCS of quarters infected with SaM to healthy quarters with DIM as a fixed predictor (as a third degree polynomial variable) are presented in Table 1. Somatic cell score was significantly higher in quarters infected with *S. agnetis, S. aureus, S. chromogenes, S. devriesei, S. haemolyticus, S, hyicus, S. simulans, S. warneri, and S. xylosus* compared to uninfected quarters (Table 1).

Least square means estimates of quarter SCS across DIM for the ten different SaM modeled as compared to healthy quarters are presented in Figure 3. Estimates for each species are presented for the observed range of DIM available from included quarter-milk samples. Infection by most SaMspecies led to elevation of quarter-milk SCS notably above the SCS of healthy quarters (Figure 3).

Predicted raw SCC for quarters infected with different SaMspecies at 91 days in milk are presented in Table 2. The highest cell count was for quarters infected with *S. warneri,* followed by *S. aureus, S. agnetis,* and *S. hyicus* (Table 2)*.* Intramammary infection with the most frequent species, *S. chromogenes,* resulted in a quarter somatic cell count of 80,376 cells/mL for a quarter of a cow at 91 DIM (Table 2).

***Discussion***

The current study describes how quarter-milk SCS varied as a result of IMI with the most frequently isolated SaM from a longitudinal, cross-sectional study of 10 certified organic dairy farms in Vermont, US. The relative distribution of various SaM and their effect on qSCC was similar to previous studies reporting data for conventionally-managed dairies. *S. chromogenes* was the most frequent species, followed by *S. aureus, S. haemolyticus,* and *S. simulans*. A large amount of variability was observed in qSCC for healthy quarters and those infected with a number of species, especially *S. chromogenes, S. haemolyticus, S. simulans,* and *S. aureus*. SCC was significantly higher in quarters infected with *S. agnetis, S. aureus, S. chromogenes, S. devriesei, S. haemolyticus, S. hyicus, S. simulans, S. warneri*, and *S. xylosus* compared to healthy quarters. The highest cell count was for quarters infected with *S. warneri*, followed by *S. aureus, S. agnetis*, and *S. hyicus*.

*S. chromogenes* was the most frequently identified SaM associated with subclinical IMI on 10 organic dairy herds in Vermont. This is consistent with other studies using genotypic methods or MALDI-TOF for speciation of SaM isolates from both conventional (De Visscher et al., 2016; Condas et al., 2017a; Rowe et al., 2019; Wuytack et al., 2020) and organic (Peña-Mosca et al., 2023) herds in various countries. In contrast to other research focused on SaM epidemiology and similar to Peña-Mosca et al. (2023), we included *S. aureus* IMI data in our analysis. This was motivated by two factors: 1) *S. aureus* has previously been identified as a pathogen of particular concern on organic dairy farms in the US (Ruegg, 2009), and 2) *S. aureus* IMI would serve as a relevant reference category for effect of IMI on SCS (in addition to healthy/negative control quarters). In agreement with Peña-Mosca et al. (2023), the second most frequently isolated SaMspecies among these ten herds was *S. aureus.* Distribution of the next most frequently found species (in order, *S. haemolyticus, S. simulans, S. agnetis,* *S. warneri*, *S.* *devriesei*) in the current study was most similar to previous work on SaM in the US and Canada (Condas et al., 2017a; Rowe et al., 2019). Interestingly, *S. equorum*, *S. cohnii,* *S. hominis,* and *M. sciuri* were all commonly-found SaM species in Belgian studies (De Visscher et al., 2016; Wuytack et al., 2020), but were infrequently found in the current study and not included in the final data set. As the farms in the current study were all certified organic dairies, the ecology of intramammary pathogens (including the diversity of SaMspecies found) could potentially differ from that of conventional farms. We suggest this is possible because, in addition to extent of antibiotic use, differences in management factors exist between conventional and organic dairies (Stiglbauer et al., 2013), and various management factors appear to affect the diversity of SaM species found (Dufour et al., 2012; Condas et al., 2017a). However, we found that the relative distribution of various SaM species in this population of small to midsize organic farms was similar to previous studies conducted on conventionally managed dairies.

In agreement with previous research describing the effect of SaM species on qSCC (using isolates from multiple herds and genotypic methods or MALDI-TOF for species identification), most of the frequently found species from this population of organic dairy farms increased qSCC above that of healthy quarters. Fry et al. (2014) also found *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. warneri,* and *S.* *hyicus* had a higher qSCC than healthy quarters, as well as *S. capitis* and *S. epidermidis,* two species which were not isolated in great enough numbers from milk samples in the current study to be included in the analysis. Isolates used in Fry et al. were a subset of a larger population from quarter-milk samples collected by the Canadian Bovine Mastitis and Milk Quality Research Network, described by Condas et al. (2017b). This larger study also found the same six SaM species previously listed increased quarter SCC above that of healthy quarters, as well as the other species identified in the current study (*S. aureus, S. agnetis*). It may be important to note that at the time of publication of Fry et al., *S. agnetis* had not yet been described as a distinct staphylococcal species; isolates of this species were likely present in milk samples included in that study, but not identified as such*.* While Condas et al. (2017b) found *S. equorum* to elevate quarter SCC above that of healthy quarters, we did not in this current study. The low number of *S. equorum* IMI observations in our study may have limited our ability to observe an effect on qSCC. Of the 17 SaM species included in Condas (2017b), *S. equorum* had the second lowest quarter SCC (40,800 cells/mL); the only species with a lower qSCC was *S. hominis*, which did not differ from healthy quarters (33,300 cells/mL). In the Canadian study, *S. succinus, S. saprophyticus, S. epidermidis, S. cohnii, M. sciuri, S. gallinarum, S. capitis,* and *S. arlettae* were also found to increase quarter SCC above that of healthy quarters; with the exception of *S. arlettae,* these species were isolated from IMI in the current study but were not present in high enough numbers to be included in the analysis. Although the scope of species included in Supré et al. (2011) was more limited, they also found that IMI due to *S. aureus, S. chromogenes, S. xylosus,* and *S. simulans* resulted in a higher SCC compared to noninfected quarters. One species not previously compared to healthy quarters in these aforementioned studies is *S. devriesei,* which we found significantly elevated quarter SCC above that of healthy quarters. As the SaM on these organic farms are under different selective pressures than those causing IMI on conventional farms, there is the potential that a given species may differ in its effect on qSCC and interaction with the host. For example, if dominant *S. chromogenes* strains differed between conventional and organic herds, the potential effect on qSCC could differ as well. Our current study does not test this hypothesis. Although the effects on quarter SCC for SaM on these organic dairies is similar to those previously described on conventional farms, comparisons between the studies should be made with caution, and the potential exists to design future studies comparing virulence factors and antibiotic resistance determinants of SaM isolates causing IMI on conventional vs. organic dairy farms.

The predicted SCC for quarters infected with *S. aureus* stayed above 200,000 cells/mL across the entire range of observed DIM (Figure 3), a cut-off which has been associated with decreased milk production (Shook, 1982; Hand et al., 2012). The ability of *S. aureus* to elevate quarter SCC above this threshold has been well-established (Supré et al., 2011; Taponen et al., 2022; Woudstra et al., 2023). Infection with *S. warneri* also resulted in a quarter SCC above 200,000 cells/mL throughout the range of observed DIM; at 91 DIM, the estimated qSCC was 395,190 cells/mL (95% CI: 148,189 - 1,053,891, Table 2), which was determined from 15 quarter observations. This extends the findings of Fry et al., where the geometric mean SCC for quarters with *S. warneri* was 233,200 cells/mL (95% CI: 90,400-601,600), from 9 quarter observations. The small number of isolates for this species likely resulted in the large 95% confidence intervals of predicted SCC for *S. warneri* seen in both studies. For two studies including larger number of observations for *S. warneri,* quarter SCC estimates stayed well below the 200,000 cells/mL cut-off (for 31 observations in Condas et al., 2017: 63,270 cells/mL, 95% CI: 42,010-95,280; for 105 observations in Taponen et al., 2022: 52,000 cells/mL, 95% CI: 38,000–71,000). In the current study, the predicted qSCC for *S. chromogenes, S. agnetis, S. hyicus, S. simulans,* and *S. xylosus* only became elevated over 200,000 cells/mL late in lactation (286, 208, 261, 270, and 281 DIM, respectively). This effect of DIM is not unexpected, given that SCC normally increases even in healthy quarters towards the tail-end of lactation (Schepers et al., 1997). While still elevated significantly above that of healthy quarters, those infected with *S. devriesei* and *S. haemolyticus* stayed below this threshold throughout the range of DIM assessed for each species.

In the observed data, SCS for quarters with an IMI due to *S. chromogenes* and *S. aureus* had significant overlap; this was similar to work by Woudstra et. al (2022), who reported quarter-level SCC by SaM on one dairy in Sweden. Additionally, Supré et al. (2011) found that *S. chromogenes*, *S. simulans*, and *S. xylosus* induced an increase quarter SCC comparable with that of *Staphylococcus aureus* for 3 farms in the Netherlands, while controlling for DIM, parity, milk production, and herd. More recent research from the same group found that the SCC from quarters with a persistent IMI due to *S. chromogenes* was comparable to SCC of quarters infected with a major pathogen such as *S. aureus* (Valckenier et al., 2021)*.* However, in our current study, this overlap in effect on SCC was no longer apparent for the least square means estimates of quarters infected with *S. aureus* and *S. chromogenes*, and accounting for the effects of DIM and repeated observations.

Within a given SaM species, there was considerable variability in the observed quarter SCC (Figure 2). This within-species variation was also observed by other studies looking at SCC by SaM species, including Fry et al. (2014) and Supré et al. (2011). Quarters with an IMI due to *S. chromogenes* had an especially wide span of observed quarter SCC in the current study, ranging from 2,000 (the lower limit of detection) to 6,100,000 cells/mL. This variability in the effect of *S. chromogenes* on quarter SCC was also noted in Valckenier et al. (2021), where quarters classified as having a transient IMI due to *S. chromogenes* had a mean SCC of 69,000 cells/mL, while those classified as having a persistent *S. chromogenes* IMI had a SCC of 351,000 cells/mL. Wuytack et al. (2020) found *S. chromogenes* to be the most prevalent NASM species causing IMI in quarters identified both as healthy (≤ 50,000 cells/mL) and infected, but with no observable clinical signs (> 50,000 cells/mL), as well as one of the three most common species in quarters exhibiting clinical signs of mastitis. Similarly, Condas et al. (2017b) found that in NASM-positive quarters, *S. chromogenes* was isolated with similar frequency from quarters classified as low-SCC (< 200,000 cells/mL), high SCC (> 200,000 cells/mL), and those with clinical mastitis. This observed diversity in the effect of *S. chromogenes* may suggest that strain type could play a role in the variable pathogenicity of NASM species, as some previous work suggests (Hyvönen et al., 2009; Åvall-Jääskeläinen et al., 2013; Naushad et al., 2019). More work exploring the possible effect of strain type while accounting for cow-level effects (i.e., immune response, DIM, parity), especially for *S. chromogenes*, is warranted to further understand this variability of observed effect on quarter SCC. As we further understand the ecology and epidemiology of individual NASM species and identify species or strains with host-adapted or contagious behavior, speciation and strain typing for NASM will be important as a part of mastitis control decision making.

A large amount of variability was also seen in the observed qSCC for healthy quarters (as defined by bacteriological status), which ranged from 2,000 (lower limit of detection) to 8,400,000 cells/mL. The presence of some relatively high quarter SCC observations in this group highlights the limitation of using culture as a method for identifying the quarter IMI status, as was recognized by Fry et al. (2014). Researchers in that study point out that the low sensitivity of bacterial culture as a test for IMI may have resulted in the presence of some undiagnosed IMI in the quarters defined as healthy. For a quarter to be considered culture negative in the current study, both milk samples were required to have either no growth at all or no significant growth on both plates. Despite the imperfect nature of bacteriological culture for determining IMI status, the median (Figure 2) and mean (Table 2) SCC for the negative control quarters was still well below that of most SaM species.

Strain typing was not carried out on all isolates of the same species causing IMI in a given quarter (to check that repeated observations of the same species was indeed a persistent infection), as our objective was to identify the effect on SCC by individual SaM species and not to characterize species-level persistence. Because finding the same NASM species in a given quarter on different occasions is likely insufficient evidence for a persistent infection (Dufour et al., 2012), it is possible that different strains of the same species have been clustered together in the analysis as repeated observations of a persistent IMI. This may introduce biases in our analysis if an unaccounted for interaction exists between persistency and effect on SCC at the strain level for some SaM species. This is a current gap in our knowledge and an opportunity for future research (De Buck et al., 2021). The majority of positive IMI quarters with repeated observations in the current study were *S. chromogenes*, which has been demonstrated to be a highly persistent intramammary pathogen (Piessens et al., 2011; Valckenier et al., 2021). In unpublished data from Fry et al. (2014), 90% of quarters where *S. chromogenes* was isolated at multiple time points were confirmed to be persistent infections. The second-most common type of IMI in the current study with repeated observations in a given quarter was *S. aureus,* an intramammary pathogen whose ability to cause persistent infections has been well described (Lam et al., 1996; Woudstra et al., 2023). Given these previous findings, we can only speculate that in our current study the majority of repeated observations of *S. chromogenes* or *S. aureus* IMI in a given quarter were persistent infections with the same strain. Notably, the inclusion of random effects for quarter and cow in the model controlled for these important host-level effects on quarter SCC.

As for any observational study using a non-probability sample, the potential exists for selection bias to have influenced the observed results. Enrolled herds were a convenience subsample who participated in a previous study, and could possibly systematically differ in some way when compared to the general population of organic dairies. Additionally, as non-probability sampling limits the external validity of a study, we would caution against making inferences from the findings beyond the source population. In 2021, there were 147 organic dairy farms in Vermont selling milk, with an average herd size of 87 cows making 6,627 kg milk/cow/year (USDA, 2022a). Herds in the current study were slightly smaller, averaging 69.5 cows per farm, but with higher-producing cows (7,999 kg milk/cow/year, estimated from DHIA records available for 8 of the 10 herds). For comparison, the average dairy cow in the U.S. produced an average of 10,885 kg of milk in 2021, and the average herd size was 316 cows (USDA, 2022b).

The species-specific effect of NASM IMI on milk yield remains somewhat inconclusive, but research to date suggests some NASM IMI may not negatively affect milk production (Tomazi et al., 2015; Valckenier et al., 2019; Gonçalves et al., 2020; Valckenier et al., 2020; Olofsson et al., 2024). At the individual animal level, treatment of these intramammary infections with antibiotics may therefore not always be warranted. At the herd level, control and prevention of NASM IMI may be an important concern. Although the increase in quarter SCC was modest for most of the NASM species observed in the current study, the widespread nature of these intramammary pathogens can still result in sizeable increases in the bulk tank SCC due to a large number of infected quarters in a herd. Schukken et al. (2009) found that the percentage contribution of NASM IMI to the total number of somatic cells in bulk tank milk was 17.9% for herds with a BTSCC less than 200,000 cells/mL, considerably greater than the contribution from infections with “major mastitis pathogens” in those herds. The consistently high quarter-level prevalence of NASM found in previous work (26%, Condas et al., 2017; 26%, De Visscher et al., 2016; 11.4%, Rowe et al., 2019; 33%, Wuytack et al., 2020) means that taken as a whole, IMI with these bacteria can still negatively affect the overall income of a dairy by preventing producers from achieving quality premiums. Schukken et al. (2009) point out that particularly in “herds striving for a low BMSCC [< 200,000 cells/mL],” where major mastitis pathogens have been controlled, IMI due to NASM are the next target to further improve udder health. These findings are even more applicable today, as the average SCC for dairies in the US continues to decline and more dairies are achieving a low BTSCC. In the US, the milk-weighted geometric mean BTSCC decreased from 227,000 cells/mL in 2009 to 171,000 cells/mL in 2019 (USDA-APHIS, 2021). The cohort of herds enrolled in this study fit the description of herds aspiring towards a low BTSCC, with an average BTSCC of 186,717 cells/mL (median = 163,583; range = 135,000-329,000).

***Conclusions***

The current study describes the species-specific effect of intramammary infection with staphylococci and mammaliicocci on quarter somatic cell count for a population of organic dairies. The diversity of SaM species observed on these 10 organic dairy herds and the species-level effect on qSCC was similar to previous studies in conventional herds. *S. chromogenes* was the most frequently found species, followed by *S. aureus, S. haemolyticus,* and *S. simulans.* Compared to culture healthy quarters, qSCC was higher in quarters infected with 9 of 10 SaM species identified. The highest cell count was for quarters infected with *S. warneri,* followed by *S. aureus, S. agnetis,* and *S. hyicus.* A large amount of variability was observed in qSCC for quarters infected with *S. chromogenes*, *S.* *haemolyticus, S. simulans,* and *S. aureus.* Although the increase in qSCC was modest for most SaM species observed, the widespread nature of these intramammary pathogens can still result in sizeable increases in bulk tank SCC.

***Notes***

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***Tables and figures***

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| --- | --- | --- | --- |
| **Table 1**. Final multivariable model describing the effect of intramammary infection with frequently isolated staphylococci and mammaliicoccion quarter somatic cell score, adjusted for days in milk at time of sampling. Data set is comprised of 2,620 quarter-day observations collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). | | | |
| *Fixed effects* | | | |
| Quarter-day IMI status | No. quarter observations | Coefficient estimate (SE) | *P*-value |
| Intercept | - | -0.03 (0.29) | 0.90 |
| *S. warneri\** | 15 | 5.18 (0.60) | < 0.001 |
| *S. aureus\** | 112 | 4.81 (0.22) | < 0.001 |
| *S. agnetis\** | 21 | 3.76 (0.45) | < 0.001 |
| *S. hyicus\** | 6 | 3.23 (0.85) | < 0.001 |
| *S. simulans\** | 35 | 3.11 (0.39) | < 0.001 |
| *S. xylosus\** | 11 | 2.96 (0.62) | < 0.001 |
| *S. chromogenes\** | 384 | 2.88 (0.12) | < 0.001 |
| *S. haemolyticus\** | 40 | 1.77 (0.31) | < 0.001 |
| *S. devriesei\** | 15 | 1.62 (0.54) | 0.003 |
| *S. equorum* | 9 | 0.12 (0.48) | 0.81 |
| Healthy (no growth) | 1972 | *Reference* | *Reference* |
| Days in milk | - | -0.003 (0.01) | 0.54 |
| Days in milk2 | - | < 0.001 (< 0.001) | 0.73 |
| Days in milk3 | - | < 0.001 (< 0.001) | 0.53 |
| *Random effects* | Variance |  | |
| Farm | 0.28 |  | |
| Cow | 1.0 |  | |
| Quarter | 0.47 |  | |
| \* Quarter somatic cell score differs from healthy quarters (*P* ≤ 0.05) | | | |

|  |  |  |
| --- | --- | --- |
| **Table 2**. Estimated quarter somatic cell count by intramammary infection status at 91 days in milk (13 weeks) for frequently isolated staphylococci and mammaliicocci and healthy (no growth) quarters. Data set used to make model estimations is comprised of 2,620 quarter-day observations collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). | | |
| Quarter-day IMI status | Estimated quarter somatic cell count (cells/mL) | 95% lower and upper confidence level (cells/mL) |
| *S. warneri* | 395,190 | 148,189 - 1,053,891 |
| *S. aureus* | 307,101 | 197,323 - 477,951 |
| *S. agnetis* | 148,437 | 69,021 - 319,232 |
| *S. hyicus* | 102,478 | 26,368 - 398,281 |
| *S. simulans* | 94,617 | 48,346 - 185,175 |
| *S. xylosus* | 84,985 | 30,798 - 234,512 |
| *S. chromogenes* | 80,376 | 56,942 - 113,454 |
| *S. haemolyticus* | 37,333 | 21,217 - 65,688 |
| *S. devriesei* | 33,513 | 13,597 - 82,599 |
| *S. equorum* | 11,855 | 5,292 - 26,556 |
| Healthy (no growth) | 10,927 | 8,056 - 14,822 |

A diagram of a flowchart

Description automatically generated

**Figure 1**.Flow diagram describing selection of final data set of quarter-day observations collected from 382 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US).

**Figure 2**. Somatic cell score for 2,260 quarter-day observations with an intramammary infection due to staphylococci and mammaliicocci and healthy (no growth) quarters. Quarter-day observations were collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). The red dotted line indicates a somatic cell score of 4. The observed data are displayed (i.e., quarters that were repeatedly positive for the same species contributed several SCC measurements). Each box contains 50% of the data for a species, the median (line), and is bounded by the 25th and 75th percentiles. The upper whisker represents the largest observation less than or equal to the 75th quartile plus 1.5 times the interquartile range, while the lower whisker represents the smallest observation greater than or equal to the 25th quartile minus 1.5 times the interquartile range.



**Figure 3**. Quarter somatic cell score least square means estimates as a function of staphylococci and mammaliicocci IMI and days in milk, compared to healthy (no growth) quarters. Data set used to make model estimations is comprised of 2,620 quarter-day observations collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). Model estimates for each species are only presented for the range of days in milk for IMI observations in the data set. Error bars represent the 95% confidence interval.