***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 culture-negative 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 potentially 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 described previously. The current study presents data from a longitudinal study of 10 certified organic dairy farms. The objective was to estimate how quarter milk somatic cell count (qmSCC) 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 qmSCC. A linear hierarchical repeated measures mixed model was used to estimate qmSCC for quarters with an IMI caused by a given SaM species, compared to culture-negative quarters. The model included days in milk at time of sampling to adjust qmSCC estimates for each SaM species. The final data set consisted of 648 quarters with an IMI due to 10 different SaMspp. and 1,972 culture-negative 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 culture-negative 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 culture-negative 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 qmSCC in this population of small to midsize organic farms was similar to previous studies. Although the increase in qmSCC was modest for most SaM species observed, the widespread presence of these intramammary pathogens could potentially contribute to sizeable increases in bulk tank SCC.

***Keywords:***

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

***Author-defined abbreviations:***

qmSCC = quarter milk somatic cell count

SaM = staphylococci and mammaliicocci

***Introduction***

Staphylococci and mammaliicocci are the predominant pathogens causing intramammary infections in dairy animals globally. This group (abbreviated as SaM), includes the major mastitis pathogen *Staphylococcus aureus* and a heterogeneous group of bacteria known as the non-*aureus* staphylococci and mammaliicocci (NASM). NASM have typically been described as minor mastitis pathogens in the literature (Griffin et al, 1977; DeBuck et al., 2021). However, for many dairy farms that have implemented modern mastitis control practices minimizing the effects of “major” pathogens such as *S. aureus*, IMI due to NASM are the leading contributor to bulk tank milk SCC on farms with good milk quality (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). However, 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). Historically, the SaM have been grouped based on coagulase test reactions as either *S. aureus* or coagulase negative staphylococci (CNS) (Schukken et al., 2009; De Buck et al., 2021). Recognizing some non-*aureus* staphylococci (NAS) are coagulase-positive or coagulase-variable, the terms CNS and NAS are not perfectly synonymous. With a recent reclassification of some staphylococcal species into the genus *Mammaliicoccus*, the term NASM entered the literature (De Buck et al., 2021). CNS identification based on coagulase testing and other phenotypic methods in previous studies may introduce uncertainty when making comparisons to recent studies using less ambiguous identification methods such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or genotypic methods (De Buck et al., 2021).

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 of a 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). In a review of the relevant literature, 7 studies were identified which describe the effect of observed species on quarter-level SCC using observations from multiple herds, where isolates were identified using MALDI-TOF MS or genotypic methods (Supré et al., 2011; Fry et at., 2014; De Visscher et al., 2016; Condas et al., 2017b; Nyman et al., 2018; Wuytack et al., 2020; Taponen et al., 2022). Most of these studies reported effect on quarter-level SCC for only a selected number of NASM species, and 4 accounted for days in milk at time of observation (Supré et al., 2011; Fry et al., 2014; Condas et al., 2017b; Nyman et al., 2018;) 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). As best as can be determined, none of these prior studies reported NASM IMI quarter milk somatic cell count (qmSCC) data from organic dairy herds in their regions. DeBuck et al. (2021) noted that while data may suggest regional differences exist in the prevalence and distribution of individual NASM species, within each region evidence of herd level differences suggest herd management influences species distribution. They concluded that additional studies are needed to better characterize these influences.

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, US organic dairies differ from conventional herds in a number of ways. These include less use of nutritional and veterinary support and less vaccination (Stiglbauer et al., 2013), and different attitudes concerning mastitis treatment and control (Ruegg, 2009). Further, in the absence of antibiotic use on organic dairies, antimicrobial susceptibility of staphylococci is higher on organic dairy farms compared to conventional farms in the US (Tikofsky et al., 2003; Pol and Ruegg, 2007; Bombyk et al., 2008). In contrast, studies from other regions have found no difference in antimicrobial resistance between organic and conventional herds (Bennedsgaard et al., 2006, Garmo et al., 2010). As noted by ,it is critical to the fact that various countries differ in their organic standards . Many European countries allow antimicrobial use in organic herds (albeit under increased constraints, such as extended withdrawal periods and strict veterinary oversight). This contrasts with the US, where all antimicrobial use is prohibited on organic farms and cattle must be removed from the herd if they are treated with unapproved substances.

Within the US, factors beyond antimicrobial use differentiate organic and conventional herds. Compared to US conventional herds, organic farms tended to milk fewer cows with lower average milk production, were more likely to use tiestall or stanchion barns, and exhibited differences in how cows were fed and watered (Zwald et al., 2004). When farms were matched for size, organic farms had cows with a higher average lactation number, fed less grain, produced less milk, utilize more access to pasture as a component of their nutritional management programs, and were less likely to regularly use a nutritionist or veterinarian (Stiglbauer et al., 2013). Further, organic and conventional farms in the US differ in their approach both to identification and management of mastitis (as reviewed in Ruegg, 2009).

At the bulk tank milk level, organic farms were more likely to be positive for *S. aureus*, but less likely to have an increased colony count (Stiglbauer et al., 2013), whereas conflicting findings have been reported for somatic cell count (SCC) (Cicconi-Hogan et al., 2014; Levison et al., 2016). At the cow level, some work found SCC was higher on organic farms (Zwald et al., 2004), while others found no difference (Hardeng and Edge, 2001; Mullen et al., 2013). A lower level of clinical mastitis has been reported for organic dairies (Hamilton et al., 2006; Richert et al., 2013; Levison et al., 2016), although this difference disappeared in Valle et al. (2007) when controlling for lower milk production by organic cows. While some research found no difference (Mullen et al., 2013), Pol and Ruegg (2007) found that the prevalence of most mastitis pathogens was higher on organic vs. conventional farms in the US.

Several of these management factors have been shown to influence the prevalence and distribution of NASM IMI in previous studies. Among Canadian herds, CNS IMI incidence was found to be associated with both bedding type and pasture access (Dufour et al., 2012), and NASM species prevalence, distribution and diversity was associated with type of housing and bedding, as well as parity (Condas et al., 2017a). Further, various herd-level management factors related to feed and water provided to dairy cows have been shown to be associated with NASM diversity (De Visscher et al., 2017; Petzer et al., 2022).

We have identified only 2 other studies using gene sequencing or MALDI-TOF MS for species identification describing NASM prevalence and diversity on US dairy herds (Jenkins et al., 2019; Peña-Mosca et al., 2023). Jenkins et al. (2019) enrolled 6 herds in 4 midwestern and western US states. The enrolled herds were relatively large conventional dairy herds (range: 1,050 to 3,600 lactating cows; average 2,230 lactating cows), presumably housed in freestalls (Jenkins et al., 2019). Peña-Mosca et al. (2023) enrolled 5 US organic herds from 4 midwestern and western states, which ranged in both size and housing characteristics: 2 herds milked either 100 or 275 cows, and the other 3 herds milked over 1,000 cows. Milk sampling occurred specifically in the post-partum period (first 21 days in milk; Peña-Mosca et al., 2023). Prevalence of *S. aureus* and non-chromogenes NASM differed between herds (Peña-Mosca et al., 2023), although care must be taken when comparing results of this study to others as NASM IMI diversity may differ immediately post-partum compared to later in lactation (De Visscher et al., 2016). Taken together, prior research justifies conducting additional descriptive observational studies of SaM epidemiology to extend our knowledge of these species under different management conditions. US organic dairy producers and advisors can benefit from an improved understanding of the SaM species specific impacts on udder health in their herds.

The current study presents data from a longitudinal observational study of 10 certified organic dairy farms in Vermont, US. Microbiological analysis of quarter milk samples to identify IMI due to SaM was conducted in parallel with determination of qmSCC. The primary objective of this study was to estimate how qmSCC 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. To contextualize the impact of NASM on qmSCC with regards to overall udder health, a secondary objective was to quantify the frequency of NASM IMI in the selected herd population.

***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 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.

*Species identification 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 species identification using MALDI-TOF MS (Microflex, Bruker Daltonics) with Flex Control software (Bruker Daltonics). The protocol for identifying bacterial isolates with MALDI-TOF MS 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 MS were identified using *tuf*gene PCR amplicon sequencing 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 species identification information, a quarter-day IMI status was assigned to each quarter observation: 1) “culture-negative,” 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 culture-negative *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 a somatic cell score (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. The variation in SCS within a quarter over time was quantified by calculating the absolute difference between the highest SCS and the lowest SCS for each individual quarter for quarters with repeated IMI over time and for culture-negative quarters.

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 culture-negative 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 culture-negative 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 3 farms actively managing deep bedded packs for composting utilize tilling to promote aerobic decomposition to break down a bedding material of dry fine wood sawdust or shavings. The 2 other farms used a “traditional” or “deep bedded pack” system, where large volumes of fresh, dry straw (or poor-quality hay) sufficient to keep cows clean and dry are added daily to a mass of bedding that accumulates over the 6–8 months cows are housed indoors. More details on bedded pack management variation on Vermont organic dairy farms can be found in Neher et al. (2002) and Jeffrey et al. (2024).

We collected 4,212 quarter milk samples from 1,536 quarters of 384 cows. Of these, 880 quarter-observations were excluded from further analyses, including: 34 quarter-observations that did not meet our definition of either having an IMI or being culture-negative, 88 quarter-observations from enrolled quarters that were non-lactating mammary glands (blind), 224 quarter-observations excluded due to a sampling error (e.g., missing cow ID, colony not selected from quarter milk culture, duplicate quarter milk sample missing), and 534 quarter-observations excluded because ≥ 1 of the 2 duplicate quarter milk samples was classified as contaminated (12.7% of total quarter-observations collected).

We collected 657 NASM isolates. Of these, 618 were identified by MALDI-TOF MS at or above our confidence cut-off value. The remaining 39 isolates were either identified with confidence to the genus level ("Staphylococcus species", n=31, 4.7%) or were unable to be identified (“No ID”, n = 8, 1.2%). The species of these 31 isolates was successfully determined by *tuf* gene fragment PCR amplicon sequencing with ≥ 98% sequence identity. Of these 31 isolates, 30 were *S. agnetis* (n=24) or *S. hyicus* (n=6), as MALDI-TOF MS was unable to differentiate these two species, and the remaining isolate was *S. pseudintermedius*. Of the 8 that were not identified by MALDI-TOF MS, 2 were identified as *S. cohnii,* 3 as *S. chromogenes*, 1 as *S. agnetis*, and 2 as *M. fleurettii*.

The initial data set included 3,332 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* (n=3), *M. sciuri* (n=2), *M. vitulinus* (n=1), *S. auricularis* (n=1), *S. capitis* (n=1), *S. cohnii* (n=1), *S. epidermidis* (n=1), *S. gallinarum* (n=1), *S. hominis* (n=1), *S. pseudintermedius* (n=1), *S. saprophyticus* (n=1),and *S. succinus* (n=1). The final data set consisted of 2,620 observations: 648 quarters with an IMI due to 10 different SaM (each causing at least 5 IMI), and 1,972 culture-negative 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 was 2.1 (2; 1-4). Thirteen percent of observations (344/2620) were a single observation contributed to the data set by a given quarter, 40% (1042/2620) came from quarters contributing 2 time points, and 45% (1182/2620) and 2% (52/2620) 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 culture-negative 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 (equivalent to ≥ 200,000 cells/mL). 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 culture-negative 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, the 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 culture-negative 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 culture-negative 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 culture-negative quarters (Table 1).

Least square means estimates of quarter SCS across DIM for the ten different SaM modeled as compared to culture-negative 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 culture-negative 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).

The median absolute difference between the minimum and maximum SCS observed for a quarter over consecutive observations of an IMI due to the same NASM species (or repeated culture-negative observations) ranged from less than 1 (equivalent to 12,500 cells/mL) to slightly greater than 2 (equivalent to 50,000 cells/mL) (Figure S1), suggesting that observed SCS values were relatively stable over time within a quarter within a IMI category.

***Discussion***

The current study describes how qmSCC varied as a result of IMI with the most frequently isolated SaM from a longitudinal study of 10 certified organic dairy farms in Vermont, US. This study also summarizes the frequency and diversity of SaM isolates associated with IMI in a convenience sample of small- to mid-sized organic dairy farms in the northeast US. The relative distribution of various SaM and their effect on qmSCC 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 qmSCC 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*.

For many species, prior CNS epidemiology and pathogenesis studies can largely be integrated with current NASM research (De Buck et al., 2021). To acknowledge that CNS and NAS classifications are not perfectly synonymous, we retain the original nomenclature (CNS or NAS) from referenced studies when individual species comparisons cannot be made.

*Effect of NASM IMI on qmSCC*

Certain comparisons with other studies are limited, as some report qmSCC results for only a subset of species, others aggregate multiple species into groups, and variations in NASM species prevalence likely exist across the different herd populations studied (Supré et al., 2011; De Visscher et al., 2016; Wuytack et al., 2020). With these caveats in mind, the effect of various SaM on qmSCC compared to culture-negative quarters in the current study was found to be similar to previous work. Least square mean estimate qmSCC was highest for quarters infected with *S. warneri*, followed by *S. aureus, S. agnetis*, and *S. hyicus*. Least square mean estimates of qmSCC were 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 culture-negative quarters. Taponen et al. (2022) found that *S. agnetis*/*S. hyicus* and *S. simulans* isolates were associated with a geometric mean qmSCC of 342,000 and 216,000 cells/mL, respectively. De Visscher et al. (2016) reported findings for a group of 3 species considered “more relevant” to udder health: *S. chromogenes*, *S. simulans*, and *S. xylosus*. This group was linked to a higher qmSCC at parturition compared to uninfected quarters, with a geometric mean of 528,000 cells/mL vs. 240,000 cells/mL (De Visscher et al., 2016). The qmSCC at parturition for quarters infected with what they described as “less-relevant” species (*S. cohnii*, *S. equorum*, *S. saprophyticus*, and *S. sciuri;* environmental “CNS” or NASM) did not differ from the qmSCC of uninfected quarters (235,000 cells/mL vs. 240,000 cells/mL; De Visscher et al., 2016).

In contrast, Condas et al. (2017b) identified 16 NAS species with a geometric mean qmSCC greater than that of uninfected quarters in a large sample of 3,561 NAS isolates from 91 Canadian herds enrolled in a study conducted by the Canadian Bovine Mastitis Research Network. These 16 species included: *S. chromogenes*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, *S. epidermidis*, *S. cohnii*, *S. sciuri*, *S. gallinarum*, *S. capitis*, *S. arlettae*, *S. warneri*, *S. saprophyticus*, *S. agnetis*, *S. equorum*, *S. succinus*, and *S. hyicus* (Condas et al., 2017b). In that study, the highest qmSCC was associated with *S. agnetis*, *S. capitis*, *S. hyicus*, *S. gallinarum*, and *S. simulans* IMI (Condas et al., 2017b). Using a subset of the data from this study conducted by the Canadian Bovine Mastitis Research Network, Fry et al. (2014) had previously identified 8 species causing subclinical IMI associated with a mean qmSCC that was significantly higher than the qmSCC of uninfected quarters. These were *S. chromogenes*, *S. simulans,* *S. xylosus*, *S. haemolyticus*, *S. epidermidis*, *S. warnerii*, *S. capitis*, and *S. hyicus*. Among these 16 species, *S. simulans*, *S. warnerii*, and *S. hyicus* were associated with the highest mean qmSCC (Fry et al, 2014)*.* In that study, the mean qmSCC of quarters infected with *S. chromogenes*, *S. haemolyticus*, and *S. epidermidis* were not significantly different from quarters infected with *S. simulans* (Fry et al., 2014). The slight differences between these two studies of Canadian herds reporting the effect of NASM species on qmSCC can likely be attributed to variations in sample sizes.

On a broader scale, variation between studies may stem from differences in sample populations, including herd and regional factors, as well as potential variation in NASM strains within species. For example, in a previous study, our research group reported differences in the frequency of *S. chromogenes* MLST strain types between Belgium and the US (Huebner et al., 2021). To a lesser extent, differences were also observed in the frequency of *S. chromogenes* MLST strain types between 2 regions in the US (Vermont and Washington; Huebner et al., 2021). These findings suggest that populations of *S. chromogenes* may be geographically distinct (Huebner et al., 2021). Particular *S. chromogenes* strains from Swedish dairy cattle differed in inflammatory response and potentially in IMI persistence at udder quarter level (Persson Waller et al., 2023). In contrast, there was no association between genetic clusters and infection phenotypes for *S. simulans* isolates causing IMI from the same region (Persson Waller et al., 2023).

In our current study, some SaM species were not isolated in great enough numbers from milk samples to be included in the qmSCC analysis. For example, Fry et al. (2014) reported quarters infected with *S. capitis* and *S. epidermidis* had qmSCC higher than that of culture-negative quarters, but these species were found infrequently in our study. In contrast, *S. aureus* and *S. agnetis* were identified to cause increased qmSCC above culture negative quarters in both this study and in Condas et al. (2017b), but not by Fry et al. (2014.) It may be important to note that at the time of species identification by Fry et al. (2014), *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 qmSCC above that of culture-negative quarters, we did not in our current study. The low number of *S. equorum* IMI observations in our study may have limited our ability to observe an effect on qmSCC. Of the 17 SaM species included in Condas (2017b), *S. equorum* had the second lowest mean qmSCC (40,800 cells/mL); the only species with a lower mean qmSCC was *S. hominis*, which did not differ from culture-negative quarters (33,300 cells/mL). In contrast to Condas et al. (2017b), and consistent with our findings, few other studies identified *S. equorum* causing increased qmSCC compared to culture-negative quarters. *S. equorum* has been described primarily as an environmental species which is associated with extramammary, parlor-associated niches (De Visscher et al., 2014; De Buck et al., 2021). Condas et al. (2017b) also found *S. succinus, S. saprophyticus, S. epidermidis, S. cohnii, M. sciuri, S. gallinarum, S. capitis,* and *S. arlettae* increased quarter SCC above that of culture-negative 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 qmSCC 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. We identified no prior studies describing the effect of *S. devriesei* on qmSCC compared to culture-negative quarters. This may be because prevalence in herds and regions previously studied was beneath the threshold to be included for analysis, or because the studies were conducted before *S. devriesei* was recognized in species identification schemes (Supré et al., 2011; Fry et at., 2014; De Visscher et al., 2016; Condas et al., 2017b; Nyman et al., 2018; Wuytack et al., 2020; Taponen et al., 2022). Our finding that *S. devriesei* significantly elevated qmSCC compared to culture-negative quarters is a novel contribution to the literature on NASM as intramammary pathogens.

The predicted qmSCC 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 qmSCC above this threshold is 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 qmSCC 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. (2014) where the geometric mean SCC for quarters with *S. warneri* IMI was 233,200 cells/mL (95% CI: 90,400-601,600) from 9 quarter observations. In Fry et al. (2014) and our current study, the small number of isolates for this species likely resulted in the large 95% confidence intervals of predicted SCC for *S. warneri*. For 2 studies including larger number of observations for *S. warneri,* qmSCC 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 our current study, the predicted qmSCC 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). Quarters infected with *S. devriesei* and *S. haemolyticus* stayed below this 200,000 cell/ml threshold throughout the range of DIM assessed, even while qmSCC due to these 2 species was elevated significantly above that of culture-negative quarters. We observed that while DIM and infection status were each significantly associated with qmSCC, the interaction between IMI and DIM was not significant. This suggests that the effect of IMI on qmSCC was stable over time, which is consistent with findings of Shook et al. (2017).

In the observed data, SCS for quarters with an IMI due to *S. chromogenes* and *S. aureus* had significant overlap (Figure 2). This was also observed by Woudstra et. al (2022), who reported quarter-level SCC by SaM on one dairy in Sweden. Supré et al. (2011) found that *S. chromogenes*, *S. simulans*, and *S. xylosus* induced an increase qmSCC comparable with that of *S. aureus* for 3 farms in the Netherlands, while controlling for DIM, parity, milk production, and herd effect. 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 a model accounting for the effect of DIM and repeated observations, the least square mean estimate of qmSCC in the current study was lower for quarters infected with *S. chromogenes* vs. *S. aureus* infected. This can be seen by comparing the model estimates presented in Figure 3 to the crude data displayed in Figure 2.

Within a given SaM species, there was considerable variability in the observed qmSCC (Figure 2). This within-species variation was reported in 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 qmSCC 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 qmSCC 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 qmSCC. As we further understand the ecology and epidemiology of individual NASM species and identify species or strains with host-adapted or contagious behavior, species identification and strain typing for NASM will be important as a part of mastitis control decision making.

*Frequency and diversity of NASM in 10 organic dairy herds in Vermont, US*

In total, 22 different species of SaM were identified to be causing IMI in this population of organic herds. *S. chromogenes* was the most frequently identified, which is consistent with other studies using genotypic methods or MALDI-TOF MS for species identification of SaM isolates from both conventional and organic herds in various countries (De Visscher et al., 2016; Condas et al., 2017a; Rowe et al., 2019; Wuytack et al., 2020; Peña-Mosca et al., 2023). In contrast to other research focused on SaM epidemiology and similar to Peña-Mosca et al. (2023) and Condas et al. (2017b), 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 served as a relevant reference category for effect of NASM IMI on qmSCC (in addition to culture-negative control quarters). In agreement with Peña-Mosca et al. (2023), the second most frequently isolated SaMspecies among these 10 organic herds was *S. aureus. S. aureus* prevalence was also second to *S. chromogenes* in the data set from 91 Canadian herds (Condas et al., 2017b). Distribution of the next most frequently found species (in order: *S. haemolyticus, S. simulans, S. agnetis,* *S. warneri*, and *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). *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 they did not meet the frequency criteria for estimating qmSCC.

De Buck et al. (2021) observed that variations in NASM species distributions can often be attributed to regional or geographic differences. However, variations in NASM species distributions between farms within the same region suggest that additional herd-level factors influence species distribution. (Dufour et al., 2012; Condas et al., 2017a; Peña-Mosca et al., 2023). Dufour et al. (2012) reported use of straw bedding increased risk for CNS IMI compared to sand or wood products, and pasture access decreased the risk compared to no outside access. Condas et al. (2017a) found that the distribution of the most prevalent species differed between tiestall, bedded pack and freestall herds, although overall NASM prevalence was similar among barn types. The farms in the current study were all certified organic dairies, which differ in a number of management practices from other types of herds. It is unclear if prior studies enrolled organic dairy farms (with the exception of Peña-Mosca et al., 2023), so comparison of our results to previous studies should be made with caution.

Prior to initiating the study, we acknowledged the possibility that the epidemiology of intramammary pathogens (including the diversity of SaM species found) in US organic herds could potentially differ from that of conventional farms described in previous studies. We suggest this is plausible because, in addition to the extent of antibiotic use, differences in management factors exist between conventional and organic dairies. Compared to US conventional herds, US organic herds are smaller, keep cows longer, have lower milk production, are more likely to use tiestall or stanchion barns, have higher prevalence of *S. aureus* IMI, utilize more pasture as a component of their nutritional management programs, use more cross-bed cows, select cows for grazing attributes, use less veterinary services, and differ in their approach to mastitis diagnostics (Ruegg, 2009; Stiglbauer et al., 2013; Cicconi-Hogan et al., 2013, Andrews et al., 2021). Some of these management factors (housing type, bedding type, and pasture access) appear to affect the diversity of SaM species (Dufour et al., 2012; Condas et al., 2017a). The prevalence of major mastitis pathogens, antimicrobial use levels, approach to mastitis diagnostics, and cattle breeds are other factors that differentiate US organic and conventional herds and may also influence NASM species prevalence. These factors have not been examined in previous studies, suggesting opportunities for additional research. Alternatively, given that herds differ in prevalence and diversity of NASM causing IMI (i.e., herd effects are important, as reviewed in De Buck et al., 2021), it is equally plausible that NASM prevalence and diversity among US organic herds is similar to the prevalence and diversity reported in prior studies of conventional herds. Our current study was not designed to compare NASM prevalence and risk factors on organic versus conventional herds. As recommended by De Buck et al. (2021), additional studies are needed to better characterize these herd factors. Future studies might enroll US organic dairy herds and conventional herds matched on variables such as herd size and pasture use (e.g., the study design of Cicconi-Hogan et al., 2013) to test for associations between NASM diversity or prevalence and variables such as major pathogen prevalence or antimicrobial use.

Dolder et al. (2017) described quarter- and cow-level risk factors for CNS IMI in 3 Swiss herds. While the sample size was too small to identify herd-level risk factors (such as housing and pasture use), they identified herd differences in species prevalence and risk factors for *S. chromogenes,* *S. haemolyticus,* and *S. xylosus* IMI including season (months) of the year. They also found coinfection with other CNS species was a significant predictor of IMI caused by *S. haemolyticus* or *S. xylosus*. They reported: “What was defined as coinfection might happen to be a sample positive for 2 different species due to teat apex or teat canal colonization” (Dolder et al., 2017, page 5661). Their study included quarters with up to 3 different *Staphylococcus* species per milk sample, and the milk isolates associated with coinfections could have originated from various combinations of sources, including IMI, the teat canal and teat apex, as well as extramammary environmental sources. Dolder et al. (2017) suggested possible mechanisms for the significant associations between 2 species observed as coinfections, including bacterial effects (combination of virulence factors and synergism in bacteria metabolism), and herd effects (poor environmental hygiene conditions). Antagonism between species colonizing the teat apex and streak canal may also influence SaM IMI risk (Mahmmod et al., 2018). It is long recognized that bacteria isolated from milk samples collected by traditional aseptic methods are not necessarily causing an IMI (Griffin et al., 1977). An important caveat is that field studies from the 1960s and 1970s frequently focused on major mastitis pathogens and ignored IMI diagnostic criteria for minor pathogens (Bramley, 1975). In more recent work, Traversari et al. (2019) set out to discriminate between IMI and teat canal colonization by collecting both milk and teat canal swab samples from 4 Swiss herds. They reported some species [*S. equorum*, *S. xylosus*, *S. sciuri* (now *Mammaliicoccus sciuri*), *S. vitulinus* (now *Mammaliicoccus vitulinus*), and *S. succinus*] were more likely to be isolated from teat canals compared to milk samples. For other species, including those frequently associated with IMI (e.g., *S. chromogenes*, *S. haemolyticus*), there was no difference in the probability of isolating from teat swabs or milk samples (Traversari et al., 2019). These findings support further research into bacterial factors and NASM interactions within the teat skin microbiome (De Buck et al., 2021).

*Study limitations and opportunities for future research*

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). As for any observational study using a non-probability sample, the potential exists for selection bias to influence the observed results. The herds enrolled in this study were a convenience subsample from participants in a previous study, and may systematically differ in certain ways from the broader population of organic dairies in Vermont, the US, or globally. Additionally, as non-probability sampling limits the external validity of a study, we caution against making inferences from the findings beyond the study population. Despite these limitations, we believe these data contribute to the general understanding of NASM epidemiology and impact on milk quality.

One limitation of this study is basing our IMI definition on bacteriological culture status, without any correction for potential IMI misclassification (Dufour et al., 2012). The collection of duplicate samples used in series to identify NASM IMI improves specificity of culture to >97% (i.e., few false positives; Dohoo et al., 2011). Despite use of this sampling scheme, it is likely that a proportion of our isolates originated from teat apex or streak canal colonization (i.e., not from an IMI), which would overestimate IMI prevalence. Adjustments for misclassification are demonstrated to be important in studies estimating risk of IMI incidence and cure (Dufour et al., 2012). Inclusion of unrecognized teat apex and streak canal colonization isolates might explain the variation observed in qmSCC for quarters defined as having an IMI. If we assume isolates colonizing the teat apex or streak canal are unlikely to cause an increase in qmSCC, our estimates of qmSCC are likely lower than the true qmSCC associated with IMI.

Similarly, the sensitivity of our diagnostic approach is estimated at approximately 42%, meaning unrecognized (false negative) SaM IMI may be included in the culture-negative group. The presence of some relatively high qmSCC observations in the culture-negative group highlights this limitation of using culture as a method for identifying the quarter IMI status, and was also recognized by Fry et al. (2014). Unrecognized IMI may partly explain some of the variability in the observed qmSCC for culture-negative quarters, which ranged from 2,000 (lower limit of detection) to 8,400,000 cells/mL. Undiagnosed IMI in the culture-negative quarters would also inflate estimates of the qmSCC for this group, thereby underestimating the true qmSCC difference between quarters with IMI and culture-negative quarters. Opportunity for future research exists to limit data sets to NASM isolates confirmed to be causing IMI. Such studies might integrate strain typing and longitudinal study designs to focus on persistent IMI as has been proposed by De Buck et al. (2021), and recently applied in a study of *S. chromogenes* and *S. simulans* (Persson Waller et al., 2023). 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 in the current study were still well below that of most SaM species.

Strain typing of isolates of the same species causing IMI in a given quarter was not performed in the current study (i.e., to check that repeated observations of the same species were 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. Finding the same NASM species in a given quarter on different occasions is likely insufficient evidence for a persistent infection (Dufour et al., 2012), and stain typing can improve our understanding of infection dynamics, duration of infection, and reduce bias which may be introduced by repeated measures (Barlow et al., 2013; Fry et al., 2014; Persson Waller et al., 2023). It is therefore possible that different strains of the same species have been clustered together in the analysis as repeated observations of a persistent IMI in the current study. This may introduce bias 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 IMI quarters with repeated observations in the current study were *S. chromogenes*, which has been demonstrated to be a 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 frequent species causing 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 qmSCC.

As the SaM on 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 qmSCC and interaction with the host. For example, 71% of Vermont organic dairy farms utilize primarily Jersey or cross-bred cattle breeds and select for cows with improved grazing phenotypes (Andrews et al., 2021). Similarly, Stiglbauer et al. (2013) reported significantly fewer US organic dairy herds in New York, Wisconsin, and Oregon used Holstein cattle compared to conventional herds in the same region. Due to breed-specific variation in immune responses to mastitis pathogens, including minor pathogens, the distinct dairy cattle genetic backgrounds on organic farms creates the possibility that bacterial strains may be exposed to different host environments for IMI on these farms (Srithanasuwan et al., 2024). Howden et al. (2023) reviewed how *S. aureus* genotypes and phenotypes shift during the process of host adaptation, from acting primarily as a colonizer to a host-adapted pathogen capable of causing persistent infections. Murphy et al. (2019) reported bovine-adapted *S. aureus* strain types commonly isolated from Irish dairy farms (ST 71, 97, 136 and 151) differ in the range of immune responses they incite, and suggested these host-pathogen interactions influence the diversity in lineages adapted to the mammary gland. Persson Waller (2023) recently reported some particular *S. chromogenes* strains are associated with persistent IMI. It is reasonable to speculate that organic dairy cattle, which may differ in their genetic background and are under different nutritional management, may provide a different immunologic environment for pathogens compared to high producing Holstein dairy cattle managed in conventional confinement systems (Rodríguez-Bermúdez et al., 2019). If dominant *S. chromogenes* strains differed between conventional and organic herds, the potential effect on qmSCC could differ as well. However, the current study was not designed to test this hypothesis. Although the effects on qmSCC for SaM on these organic dairies is similar to those previously described on conventional farms, comparisons between the studies should be made with caution. The potential exists to design future studies comparing 1) SaM species prevalence and diversity, 2) host-adaptation and strain specific risk factors, and 3) virulence factors and antibiotic resistance determinants of SaM isolates causing IMI under different management systems. These studies would be challenging, requiring large populations of cattle in a longitudinal study design.

Another limitation of this work may be the measurement of qmSCC from frozen milk samples. Barkema et al. (1997) reported that freeze-thaw of milk samples reduced SCC measurements compared to fresh milk samples, although there was limited effect when SCC is used as an indicator of inflammation at a 200,000 threshold. Previous work reporting the effect of NASM IMI on qmSCC have also used frozen samples (Fry et al., 2014; Condas et al., 2017b). Estimates of qmSCC from the current study may be slightly reduced, which should be recognized when making comparisons to other studies measuring SCC from fresh or preserved milk. As all samples in our study were all handled in the same manner, any influence of freezing would be the same across samples and comparisons between SaM species and culture-negative milk samples would be unaffected.

Milk yield data was not collected from enrolled cows, so are unable to estimate the impact of NASM IMI on milk yield in these herds. 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 herd level, control and prevention of NASM IMI may be an important concern. Although the increase in qmSCC was modest for most of the NASM species observed in the current study, the widespread nature of these intramammary pathogens still has the potential to 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 that might aspire to achieve a lower 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 qmSCC was similar to previous studies. *S. chromogenes* was the most frequently found species, followed by *S. aureus, S. haemolyticus,* and *S. simulans.* Compared to culture-negative quarters, qmSCC was higher in quarters infected with 9 of 10 SaM species analyzed. 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 qmSCC for quarters infected with *S. chromogenes*, *S.* *haemolyticus, S. simulans,* and *S. aureus.* Although the increase in qmSCC 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***

|  |  |  |  |
| --- | --- | --- | --- |
| **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 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 |
| Culture-negative | 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 culture-negative 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 culture-negative 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 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 |
| Culture-negative | 10,927 | 8,056 - 14,822 |



**Figure 1**.Flow diagram describing selection of final data set of quarter-day observations collected from 382 cows during a longitudinal 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 culture-negative quarters. Quarter-day observations were collected from 1,272 quarters belonging to 360 cows during a longitudinal 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 culture-negative 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 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.



**Figure S1**. Variation in somatic cell score (SCS) for 898 quarters with consecutive observations (≥2) repeatedly positive for an IMI due to the same NASM species or repeatedly identified as culture-negative (no growth). The absolute difference was found between the minimum and maximum SCS observed for a quarter over consecutive observations of an IMI due to the same NASM species (or repeated observations of no growth), and is presented as variation in SCS. 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). 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.