***Interpretive summary***

Variation in distribution and diversity of *Staphylococcus* species causing intramammary infections in dairy cattle is associated with different management practices. The objective of the current study was to identify which *Staph*. species are most relevant to udder health for organic dairies, by exploring how quarter somatic cell count (SCC) varied as a result of infection with the most frequently isolated species. Compared to culture negative quarters, SCC was higher in quarters infected with 9 of 10 *Staph.* species. Although the increase in SCC was modest for most species observed, their widespread nature can still result in increased bulk tank SCC.

***Running head:***

Staph. affecting udder health on organic dairies

***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 non-*aureus* staphylococci and mammaliicocci (NASM) species causing intramammary infections (IMI) 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. No previous studies have explored the species-specific effect on quarter somatic cell count of NASM for a population of certified organic dairies. 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 *Staphylococcus* species. Aerobic culture of quartermilk samples to identify IMI was conducted in parallel with determination of qSCC. A linear hierarchical repeated measures mixed model was used to compare somatic cell scores associated with quarters identified to have IMI with a given *Staph.* species to culture negative quarters, and included days in milk at time of sampling to adjust estimates of the *Staph.* species and qSCC association. The final data set consisted of 648 quarters with an IMI due to 10 different *Staph.* sp. and 1,972 negative quarters. *S. chromogenes* was the most commonly found species, followed by *aureus, haemolyticus,* and *simulans.* A large amount of variability was observed in the somatic cell score for negative quarters and those infected with a number of *Staph.* species, especially *S. chromogenes* and *aureus.* Somatic cell score was significantly higher in quarters infected with *S. agnetis, aureus, chromogenes, devriesei, haemolyticus, hyicus, simulans, warneri, and xylosus* compared to uninfected quarters. The highest cell count was for quarters infected with *S. warneri,* followed by *aureus, agnetis,* and *hyicus.* The relative distribution of various *Staph.* species and their effect on qSCC in this population of small to midsize organic farms was similar to previous studies describing conventionally-managed dairies. Although the increase in qSCC was modest for most NASM 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. This group includes the major mastitis pathogen *Staph. aureus*, and a heterogeneous group of bacteria known as the non-*aureus* staphylococci and mammaliicocci (NASM). For many dairy farms that have implemented modern mastitis control practices minimizing the effects of “major” pathogens such as *Staph. aureus*, the leading contributor to bulk tank milk SCC on farms with good milk quality is mammary gland infections due to NASM (Schukken et al., 2009). NASM cow-level prevalence in one U.S. study was 71% (Jenkins et al., 2019), and quarter-level prevalences of 11, 26, 21, and 33% have been reported in Canada, the U.S., and Belgium (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, NASM intramammary infections (IMI) likely 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 the ability of NASM to increase somatic cell count (SCC) is well-established (Supré et al., 2011; Tomazi et al., 2015; Condas et al., 2017b; Valckenier et al., 2019), as well as their ability to persist for long periods of time in the udder (Piessens et al., 2011; Nyman et al., 2018; Valckenier et al., 2021).

NASM are 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 species 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, only 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 (DIM) 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). No previous studies have identified which *Staph.* species are most relevant to udder health by describing the species-specific effect on SCC for a population of certified organic dairies. Although similar overall in many aspects, organic and conventional dairies differ significantly in a number of ways both in management (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 U.S. (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 pressure between conventional and organic farms, potentially resulting in differences in virulence and impact on SCC.

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

***Materials and methods***

*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 (U.S.) 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 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. Participating herds milked an average of 69.5 cows (median: 70; range: 44-105) of various breeds. Five farms housed cows in a tiestall bedded with wood shavings, and 5 utilized a bedded pack system (3 actively managed for composting, 2 static). Three visits were completed at 8 farms, with 1 herd sampled twice and 1 herd 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). Around the time of the first farm visit, herd records were captured from the record processing center working with each of 9 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 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 in the herd 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). 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. 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 quartermilk samples were sent to the Vermont State Agricultural and Environmental Laboratory, where samples were thawed at time of processing and quarter-level somatic cell count was determined using flow cytometry (Somacount FC, Bentley Instruments).

*Aerobic culture of milk samples*

Standard aerobic bacteriological culture of quartermilk samples was performed in duplicate within 24 hours of collection to identify the intramammary infection status of each quarter. 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 plastic inoculating loops. Plates were then incubated in aerobic conditions at 37°C before being read at approximately 48 hrs. A quarter was considered positive for an IMI when ≥ 1 CFU (100 CFU/mL) of a particular isolate was identified with the same morphology on both plates (interpretation in series; Dohoo et al., 2011). A quarter was considered negative when there was no significant growth on either plate (i.e., no growth on both plates, or no significant growth on one plate and no growth on the other plate, or no significant growth on both plates and morphology of isolates on each plate was different). Samples that did not fit into either of these two definitions (positive or negative for IMI) were excluded from further analysis. Interpretation of duplicate quartermilk samples in series results in decreased sensitivity but higher specificity for identifying non-*aureus* staphylococci intramammary infections as compared to a single sample (Dohoo et al., 2011). This approach was chosen to maximize the specificity of culture to identify quarters as positive for a non-*aureus* staphylococci IMI (i.e., minimize false positives). Collection of a large number of samples in the field under time pressure and occasionally with minimally trained personnel resulted in a moderately high rate of contamination (13%). Quartermilk samples were classified as contaminated if more than 2 different morphologically distinct isolates grew on a plate. If either one of the two samples or both samples were classified as contaminated, that quarter was removed from analysis.

*Speciation of bacterial isolates*

Isolates cultured from quarters meeting the IMI definition were selected and grown in isolation 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, 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 species-level identification and < 1.7 was classified as inconclusive. Suspect staphylococci 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).

*Selection and description of data set*

The initial data set included 3,331 quarter observations where the bacteriological status of a quarter could be determined. Any *Staph.* species associated with < 5 quarter observations of IMI did not meet our criteria for a frequently observed species and were excluded from further analysis. Quarters were then selected that: 1) had a subclinical IMI due to any frequently observed *Staph.* species (≥ 5 observed IMI) in pure culture, *or* was a culture negative quarter; 2) was collected from a cow ≤ 305 days in milk at time of observation; and 3) had an associated quarter-level somatic cell count measurement (Figure 1)*.*

*Statistical analysis*

Quarter somatic cell counts, intramammary infection status, cow parity and days in milk 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-level somatic cell count was converted to somatic cell score [log2(quarter somatic cell count/1000) + 3] (SCS) in order to address the non-normal distribution of SCC data.

A linear hierarchical repeated measures mixed model was fitted to the data set in order to compare SCS of quarters with *Staph.* species intramammary infections (in pure culture) to culture negative quarters. The “lme” function of the “nlme” package was used to build this model, in which the SCS for each quarter observation was the outcome variable, and *Staph.* species causing IMI (with culture negative quarters as the reference value) was the fixed predictor variable. The number of days in milk at time of sampling was included in the model to adjust the estimates of the *Staph.* species and quarter SCS association for confounding by this variable. 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 was:

SCS*ijkl* = β0 + β1 *Staph.* species*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 *Staph.* species, and DIM as a cubic term (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). Biologically plausible interactions were investigated between IMI status, SCS, and parity variables. 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***

The initial data set included 3,331 quarter-level observations, with 22 different species of staphylococci and mammaliicocci identified. *Staphylococcus* and *Mammaliicoccus* 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 intramammary infection due to 10 different *Staph.* sp. (each with at least 5 associated IMI observations), 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 sampled 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.

*Staph. chromogenes* was the most commonly found species (59% of IMI quarter observations), followed by *Staph. aureus* (17%)*, Staph. haemolyticus* (6%)*,* and *Staph. simulans* (5%)*.* A large amount of variability was observed in the SCS for culture negative quarters and those infected with a number of different *Staph.* species, especially *S. chromogenes* and *S. aureus* (observed quarter SCS data presented in Figure 2). The observed SCS for *S. chromogenes* IMI ranged from -2.64 to 8.93 (median: 3.26; equivalent to 2,000 to 6.1 million cells/mL), with 29.7% of observations of having an SCS ≥ 4. The observed SCS for *S. aureus* IMI ranged from 0.64 to 10.49 (median: 5.93; equivalent to 8,000 to 18 million cells/mL), with 87.5% of observations of having an SCS ≥ 4.

The final model comparing SCS of quarters infected with *Staph.* species to culture negative quarters adjusted for days in milk is 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 (P ≤ 0.05; Table 1). The interaction between IMI status and DIM was not significant (P = 0.42). The effect of parity on SCS was visualized using the raw data, and appeared to have a positive linear relationship with SCS. When SCS was plotted as function of IMI status by parity using the raw data, most bacterial species (with the exception of *S. hyicus,* n = 6 observations) had a relatively constant effect on SCS regardless of parity. A model with an interaction term between IMI status and parity found that the interaction between IMI status and parity was not significant (P = 0.86), but parity and bacterial species separately were both significant predictors of SCS (P < 0.0001). A model was attempted with a three-way interaction term between DIM (3-degree polynomial term), IMI status, and parity, but would not converge due to complete data separation. An additive model with DIM (3-degree polynomial term), IMI status, and parity found all three variables to be statistically significant (P < 0.0001) predictors of SCS. When compared to the model with only DIM and IMI status, the model including parity changed the coefficients for each *Staph.* sp. group by ≤ 5%, and standard errors by ≤ 1%. As the effect of parity was the same across all groups of IMI status, and the impact of its inclusion was minimal on the coefficients of the variable of interest, only results from the model including DIM (3-degree polynomial term) and IMI status on quarter SCS are presented in the interest of simplicity.

Least square means estimates of quarter SCS across DIM for the ten different *Staph*. species 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 quartermilk samples. Infection by most *Staph.* species elevated quarter SCS notably above the SCS for no growth quarters (Figure 3).

Predicted raw somatic cell counts for quarters infected with different *Staph.* species 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 commonly found species, *S. chromogenes,* resulted in a quarter somatic cell count of 80,376 cells/mL for a cow 91 days in milk (Table 2).

***Discussion***

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

*S. chromogenes* was the most frequently identified *Staph.* species 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 staphylococcus 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 work focused on NASM 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 U.S. (Ruegg, 2009), and 2) *S. aureus* IMI would serve as a relevant reference category for effect of IMI on SCS (in addition to culture negative control quarters). In agreement with Peña-Mosca et al. (2023), the second most frequently isolated *Staph.* species among these ten herds was *S. aureus.* Distribution of the next most frequently found *Staph.* species (in order, *S. haemolyticus, S. simulans, S. agnetis,* *S. warneri*/*S.* *devriesei*) in the current study was most similar to previous work on NASM in the U.S. and Canada (Condas et al., 2017a; Rowe et al., 2019). Interestingly, *S. equorum*, *S. cohnii,* *S. hominis,* and *M. sciuri* were all commonly-found NASM 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 are all certified organic dairies, the ecology of intramammary pathogens (including the diversity of *Staph.* species 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 NASM species found (Dufour et al., 2012; Condas et al., 2017a). However, we found that the relative distribution of various *Staph.* species in this population of small to midsize organic farms was similar to previous studies describing conventionally managed dairies.

Similarly, as the *Staph.* species on these organic farms are under different selective pressures than those causing IMI on conventional farms, there was the potential that a given species may differ its effect on SCC and interaction with the host. For example, it is unknown if dominant *S. chromogenes* strains differ between conventional and organic herds. Although we did not test this specific hypothesis, we found no evidence that a given species may vary in effect on SCC in the current study. Similar to previous work describing the effect of different *Staph.* species on quarter SCC (using isolates from multiple herds and genotypic methods or MALDI-TOF for identification), most of the frequently found species from this population of organic dairy farms increased qSCC above that of culture negative quarters. Although differences in study design preclude direct comparison of species-level effect on SCC across publications, similar general trends are observed. Fry et al. (2014) also found *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. warneri,* and *S.* *hyicus* had a higher quarter SCC than negative control 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 quartermilk 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 NAS species previously listed increased quarter SCC above that of culture negative quarters, as well as other staphylococci species included in the current study (*S. aureus, S. agnetis*). While Condas et al. (2017b) found *S. equorum* to elevate quarter SCC above that of negative quarters, the current study did not. Of the 17 NAS species they included, *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 culture negative 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 negative control 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 than noninfected quarters. One species not previously compared to negative quarters in these aforementioned studies is *S. devriesei,* which we found significantly elevated quarter SCC above that of negative control quarters. Although the effects on quarter SCC for *Staph.* species on these organic dairies is similar to those previously described on conventional farms, the potential exists for future work comparing virulence factors and antibiotic resistance determinants of NASM 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 based off 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), which was based off 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 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 culture negative quarters, those infected with *S. devriesei* and *S. haemolyticus* stayed below this threshold throughout the range of DIM assessed for each species. A readily-available, reliable bench-top test has not yet been developed for differentiating NASM species. With the exception of larger milk quality labs and research settings, the best current methods of speciation for NASM (MALDI-TOF, PCR) are not widely used due to a high cost and technological barrier. Currently, most NASM species are only able to be lumped together as “non-*aureus* staphylococci” by milk quality labs without the resources or infrastructure to speciate isolates (e.g., on-farm culture, veterinary practices), even though it is established that some species are more relevant to udder health than others. Future work towards developing more readily available methods of speciation may better inform treatment decisions for producers, allowing them to treat or cull animals with infections due to more problematic NASM and withhold treatment for those of less concern.

Although the increase in quarter SCC was modest for most of the NAS species observed in the current study, the widespread nature of these intramammary pathogens can still result in sizeable increases in the bulk tank somatic cell count due to a large number of quarters infected in a given 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, intramammary infections with these bacteria can still negatively affect the overall income of a dairy by preventing producers from achieving quality premiums. Schukken et al. point out that in particular, in “herds striving for a low BMSCC [< 200,000 cells/mL],” where major mastitis pathogens have already 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 somatic cell count for dairies in the U.S. continues to decline and more dairies are achieving a low BTSCC. In the U.S., the milk-weighted geometric BTSCC mean 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).

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 *Staph.* species 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 (Valckenier et al., 2021) 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.* However, in the 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*, which accounted for the effects of DIM and repeated observations.

Within a given *Staph.* species group, 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 *Staph.* 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 NAS species causing IMI in quarters identified both as healthy (≤ 50,000 cells/mL) and subclinically infected (> 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 NAS-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 NAS 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 culture negative quarters included in the study, 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 likely highlights the limitation of using bacterial culture as a method for identifying IMI, as was pointed out 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 negative control quarters. The definition for an IMI in the current study (duplicate milk samples interpreted in series) results in an even lower sensitivity than used by Fry et al., which may compound this issue. However, in order 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, which is also a fairly strict definition. Despite this limitation, the median (Figure 2) and mean (Table 2) SCC for the negative control quarters was still well below that of most NAS 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 NASM species and not to characterize species-level persistence. As 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 *Staph.* 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 *Staph.* 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). Based on previous findings, we can only speculate that the majority of repeated observations of *S. chromogenes* or *S. aureus* IMI in the current study 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.

***Conclusions***

The current study is the first to describe the species-specific effect of intramammary infection with staphylococci on quarter somatic cell count for a population of organic dairies. As variation in *Staph.* species diversity is associated with differences in management practices, the relative distribution of species could potentially differ between organic and conventional dairies. However, the diversity of *Staph.* species observed and their effect on qSCC was similar to previous studies. *S. chromogenes* was the most commonly found species, followed by *aureus, haemolyticus,* and *simulans.* Compared to culture negative quarters, qSCC was higher in quarters infected with 9 of 10 *Staph.* species identified. The highest cell count was for quarters infected with *S. warneri,* followed by *aureus, agnetis,* and *hyicus.* A large amount of variability was observed in qSCC for quarters infected with *S. chromogenes* and *aureus.* Although the increase in qSCC was modest for most NASM species observed, the widespread nature of these intramammary pathogens can still result in sizeable increases in bulk tank SCC.

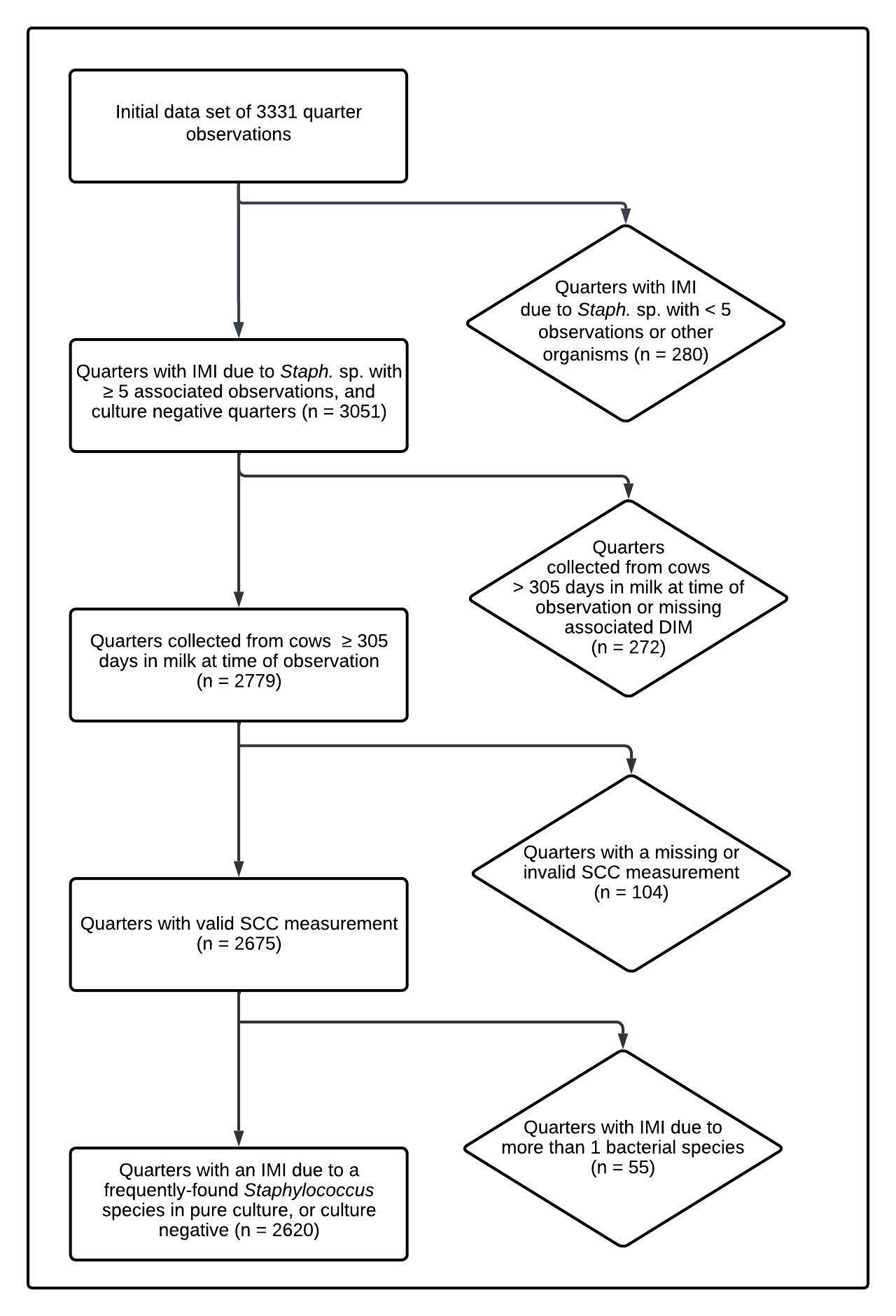


Figure 1.Flow diagram describing selection of final data set.

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| Table 1. Final multivariable model describing the effect of intramammary infection with frequently isolated *Staphylococcus* species on quarter somatic cell score, adjusted for days in milk at time of sampling. | | | |
| *Fixed effects* | | | |
| *Staphylococcus* sp. | No. quarter observations | Coefficient estimate (SE) | *P*-value |
| Intercept | - | -0.03 (0.29) | 0.90 |
| No growth | 1972 | *Reference* | *Reference* |
| *S. agnetis\** | 21 | 3.76 (0.45) | < 0.00001 |
| *S. aureus\** | 112 | 4.81 (0.22) | < 0.00001 |
| *S. chromogenes\** | 384 | 2.88 (0.12) | < 0.00001 |
| *S. devriesei\** | 15 | 1.62 (0.54) | 0.003 |
| *S. equorum* | 9 | 0.12 (0.48) | 0.81 |
| *S. haemolyticus\** | 40 | 1.77 (0.31) | < 0.00001 |
| *S. hyicus\** | 6 | 3.23 (0.85) | 0.0001 |
| *S. simulans\** | 35 | 3.11 (0.39) | < 0.00001 |
| *S. warneri\** | 15 | 5.18 (0.60) | < 0.00001 |
| *S. xylosus\** | 11 | 2.96 (0.62) | < 0.00001 |
| Days in milk | - | -0.003 (0.01) | 0.54 |
| Days in milk2 | - | 0.00001 (0.00004) | 0.73 |
| Days in milk3 | - | < 0.00001 (< 0.00001) | 0.53 |
| *Random effects* | Variance |  | |
| Farm | 0.28 |  | |
| Cow | 1.0 |  | |
| Quarter | 0.47 |  | |
| \* Quarter somatic cell score differs from negative controls (P ≤ 0.05) | | | |

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| --- | --- | --- |
| Table 2. Estimated quarter somatic cell count by intramammary infection status at 91 days in milk (13 weeks) for frequently isolated *Staphylococcus* species and culture negative quarters. [which one do we like better?] | | |
| *Staphylococcus* sp. | Estimated quarter somatic cell count (cells/mL) | 95% lower and upper confidence level (cells/mL) |
| No growth | 10,927 | 8,056 - 14,822 |
| *S. agnetis* | 148,437 | 69,021 - 319,232 |
| *S. aureus* | 307,101 | 197,323 - 477,951 |
| *S. chromogenes* | 80,376 | 56,942 - 113,454 |
| *S. devriesei* | 33,513 | 13,597 - 82,599 |
| *S. equorum* | 11,855 | 5,292 - 26,556 |
| *S. haemolyticus* | 37,333 | 21,217 - 65,688 |
| *S. hyicus* | 102,478 | 26,368 - 398,281 |
| *S. simulans* | 94,617 | 48,346 - 185,175 |
| *S. warneri* | 395,190 | 148,189 - 1,053,891 |
| *S. xylosus* | 84,985 | 30,798 - 234,512 |

|  |  |  |  |
| --- | --- | --- | --- |
| Table 2. Estimated raw quarter somatic cell count by intramammary infection status at 91 days in milk (13 weeks) for frequently-isolated *Staphylococcus* species and culture-negative quarters. [which one do we like better?] | | | |
| *Staphylococcus* sp. | Estimated quarter somatic cell count (× 1,000 cells/mL) | 95% lower confidence level (× 1,000 cells/mL) | 95% upper confidence level (× 1,000 cells/mL) |
| No growth | 10.9 | 8.1 | 14.8 |
| *S. agnetis* | 148.4 | 69 | 319.2 |
| *S. aureus* | 307.1 | 197.3 | 478 |
| *S. chromogenes* | 80.4 | 56.9 | 113.5 |
| *S. devriesei* | 33.5 | 13.6 | 82.6 |
| *S. equorum* | 11.9 | 5.3 | 26.6 |
| *S. haemolyticus* | 37.3 | 21.2 | 65.7 |
| *S. hyicus* | 102.5 | 26.4 | 398.3 |
| *S. simulans* | 94.6 | 48.3 | 185.2 |
| *S. warneri* | 395.2 | 148.2 | 1,053.9 |
| *S. xylosus* | 85 | 30.8 | 234.5 |

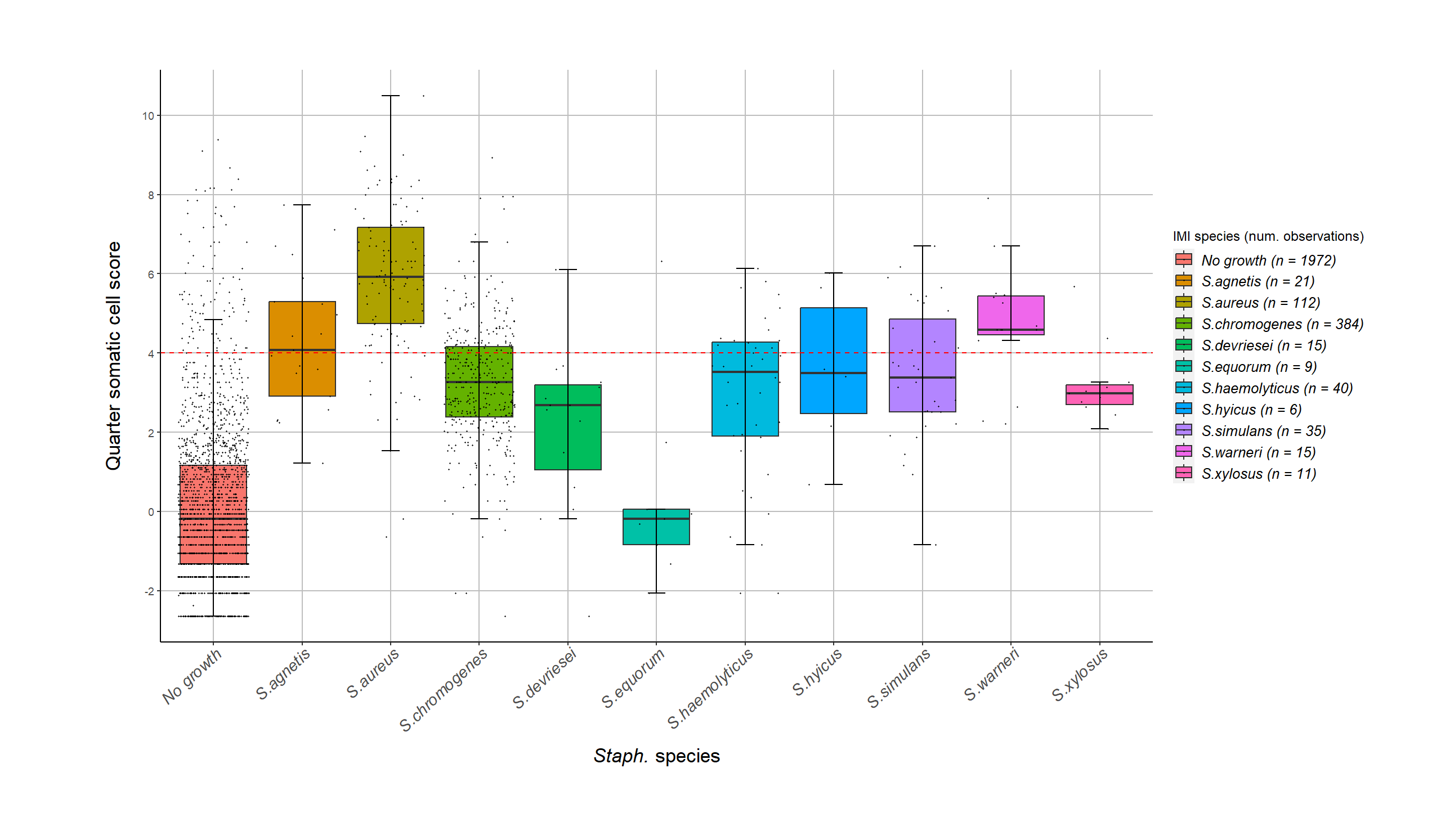


Figure 2. Somatic cell score for quarters with an intramammary infection due to *Staphylococcus* species and culture negative control quarters. The red dotted line is at a somatic cell score of 4. The observed data are displayed (i.e., quarters that were repeatedly positive for the same species contributed with 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 *Staph.* species IMI and days in milk, compared to culture negative quarters. 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.

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