***Chapter 6: Summary, future directions, general conclusions and overall significance***

***6.1 Summary***

The overall objective of this dissertation research was to better understand the epidemiology of the most relevant pathogens causing IMI in organic dairy cows in Vermont. In particular, this work generated foundational knowledge about staphylococci species causing mastitis on organic dairies. Bacteria belonging to the genus *Staphylococcus* are the predominant pathogens causing intramammary infections (IMI) in dairy animals, on organic farms in Vermont, across the US, and for all farm types globally.

Chapter 1 of this dissertation describes the findings of an observational study of organic dairy herds which was undertaken to identify differences in udder health outcomes between herds using different housing systems. Previous research had shown that tiestall barns were the most frequently used housing type for lactating dairy cattle on organic farms in Vermont, followed by freestall barns. For most of the udder health metrics and the two udder hygiene measures studied, herds using a bedded pack system either performed slightly better or were equivalent in comparison to the most commonly used facility types in Vermont.

The diversity of bacterial species most frequently causing IMI on 10 organic farms in Vermont were then identified in a longitudinal study, for all pathogen types in Chapter 5 and specifically staphylococci and mammaliicocci in Chapter 3. Species from the genus *Staphylococcus* were responsible for the majority of mastitis in these herds. Overall, the majority of IMI were caused by all non-*aureus* staphylococci and mammaliicocci (NASM) combined. At the species level, *Staph. chromogenes* was the leading cause of IMI for both facility types, followed by *Strep. uberis*, *Staph. aureus*, and *Staph. haemolyticus*. Quarter-level prevalence of IMI by pathogen was similar between bedded pack and tiestall farms, with slight variability in the most prevalent species. For the bedded pack farms, the most commonly identified pathogens were *Staph. chromogenes,* followed by *Strep. uberis*, *Staph. haemolyticus,* and then *Staph. aureus.* For the tiestall farms, the most commonly identified pathogens were *Staph. chromogenes,* followed *Staph. aureus*, *Strep. uberis*, and then *Corynebacterium* spp*.*

Chapter 3 focused specifically on IMI caused by staphylococci and mammaliicocci in the longitudinal study. Within this group of species, *Staph. chromogenes* was the most frequently found, followed by *S. aureus*. The diversity of staphylococcal species observed and the species-level effect on inflammatory reaction (as measured by somatic cell count, or SCC) was similar to conventional herds. Compared to healthy quarters, SCC was higher in quarters infected with nine of 10 staphylococcal species identified. Although increase in SCC was modest for most staphylococcal species, their widespread nature can still result in sizeable increases in bulk tank SCC. A large amount of variability was observed in SCC associated with *Staph. chromogenes* IMI, with some causing an elevation comparable to that of major mastitis pathogens and others similar to healthy quarters.

The majority of *Staph. chromogenes* IMI observed in the longitudinal study were persistent. Chapter 4 describes the findings of a study to better understand the epidemiology of this subgroup of persistent *Staph. chromogenes* IMI. Two categories selected from persistent *Staph. chromogenes* IMI: those associated with a consistently high SCC and those with a consistently low SCC. Representative bacterial isolates from these IMI were submitted for whole genome sequencing and strain-typed. Particular strain types of *Staph. chromogenes* were not found to be associated with either SCC category. Ten different strain types were identified, four of which were newly-described. The only antimicrobial resistance gene identified was *blaZ*, encoding for resistance to penicillin in a third of the study isolates. Neither overall number of virulence factors nor *blaZ* carriage was found to be a significant predictor of SCC category. *blaZ* carriage, number and type of virulence factor instead appeared to be a function of strain type (Table 6.1).

***6.2 Directions for future research***

*6.2.1 Further work exploring associations between management factors and udder health for bedded pack farms*

The biggest limitation encountered in the cross-sectional study described in Chapter 2 was the relatively small number of farms included for each of the three facility types of interest. This limited statistical power when comparing udder health and hygiene metrics between tiestalls, freestalls, and bedded pack farms. Further complicating analysis between farms was how similar milk quality and udder health were among all 21 enrolled herds; most herds were below benchmark cutoffs for the number of cows developing new IMI per month, cows with chronic IMI month to month, proportion of cows with an elevated SCC, and all bulk tank somatic cell counts were all fairly low (54,000-250,000 cells/mL). A related limitation is that well-established mastitis control practices were widely adapted by all participating herds, so we were unable to analyze associations between certain practices and bulk tank milk quality, udder health, and hygiene. The potential still exists for future studies with a larger number of farms enrolled to further characterize milk quality and udder health on bedded pack systems in the Northeastern US climate. By enrolling farms from a larger geographic area (vs. only Vermont), future studies may be able to include a larger number of bedded packs, with more variation in milk quality and mastitis rates. This could increase the statistical power needed to identify particular management factors which are beneficial or detrimental for these farms specifically. Although we were limited by statistical power for our cross-sectional study, our data could be used to inform new hypotheses and power calculations for future study design.

*6.2.2 Further work exploring virulence potential for Staph. chromogenes isolates from the current study*

In Chapter 4, the total number of VF identified for each *Staph. chromogenes* isolate was not a significant predictor of whether that isolate would belong to the persistently high or persistently low SCC IMI category. In a linear regression with all virulence factors considered together, Naushad et al. (2019) did not find that an increase in the overall number of virulence factors for a NASM isolate was associated with an increase in the logSCC of the associated IMI. However, when stratified by type of virulence category, the presence of each additional toxin gene for a NASM isolate was associated with a 0.024 increase in logSCC of the associated IMI (Naushad et al., 2019). Similarly, in a logistic regression with ordinal categories for IMI severity (low SCC, medium SCC, high SCC, and clinical mastitis), an overall increase in the number of virulence factors was not associated with increased severity of an IMI (Naushad et al., 2019). In a regression analysis with VF stratified by type of virulence category, the presence of each additional virulence factor gene associated with host immune evasion increased the odds of having a more severe immune response by 0.074 (Naushad et al., 2019). For the 30 *Staph. chromogenes* isolates included in Chapter 4, it would be worth repeating the analysis of possible associations between number of virulence factors and SCC category, but stratified by the different groupings of virulence gene by functional category. Naushad et al. (2019) define five separate functional groupings for virulence genes (adherence, exoenzymes, host immune evasion, iron metabolism, and toxins), and analysis of number of virulence genes by functional group may be associated with SCC category even though overall number of virulence genes was not. Furthermore, Naushad et al. (2019) applied various clustering approaches in order to determine whether particular virulence distributions had any association with SCC category or occurrence of clinical mastitis, as well as relationships between the virulence factors from five categories stratified by IMI severity (low SCC, medium SCC, high SCC, and clinical mastitis). Although these statistical methods were beyond the scope of the current dissertation, cluster analyses of the study isolates may reveal interesting associations between different virulence factors, or patterns of virulence factors distinct to either the persistently high or low SCC IMI group.

The gene *coa*, encoding for the staphylocoagulase enzyme, was identified in two *Staph. chromogenes* isolates belonging to ST25. *coa* has not been widely reported to be present in *Staph. chromogenes* of bovine IMI origin. It would be interesting to screen more *Staph. chromogenes* from the current study for presence of *coa,* whether by whole genome sequencing or PCR. Both isolates with *coa* belonged to ST25, so identifying more isolates with MLST and screening for *coa* could identify if carriage of this notable staphylococcal virulence factor was more likely isolates belonging to this particular strain type (ST). Additionally, performing a coagulase test for *coa* positive isolates in this collection could yield interesting insight between carriage of this gene and phenotypic ability to clot serum *in vitro.*

*6.2.2 Further work exploring antimicrobial resistance for Staph. chromogenes isolates from the current study*

Ten of the thirty isolates *Staph. chromogenes* isolates described in Chapter 4 were positive for carriage of the *blaZ* gene with *in silico* analysis of data from whole genome sequencing. *blaZ* encodes a β-lactamase enzyme which hydrolytically destroys β-lactam antibiotics, and is the primary determinant of phenotypic resistance to benzylpenicillin in staphylococci (Pinho, 2008). However, carriage of a particular resistance gene does not always translate into phenotypic resistance. Inconsistencies exist between phenotypic and genotypic resistance results, due either to 1) detection of phenotypic resistance in the absence of expected genotypic determinants, or 2) phenotypic susceptibility despite the presence of genotypic determinants. For staphylococciassociated with bovine mastitis, both of these types of discrepancies have been reported for penicillin resistance (Sampimon, 2009; Taponen et al., 2023). In a study by Taponen et al. (2023) comparing methods of testing for β-lactamase mediated resistance, overall agreement between phenotypic and genotypic resistance tests was moderate to substantial for staphylococci from bovine IMI. However, some inconsistencies were found between phenotypic susceptibility by disk diffusion method, the nitrocefin test to assess β-lactamase production, and PCR to detect the presence of the *blaZ, mecA*, and *mecC* genes encoding the β-lactamase gene.

Performing phenotypic antibiotic susceptibility testing for the 30 *Staph. chromogenes* isolates described in Chapter 4 could further inform agreement between genotypic and phenotypic susceptibility of bovine staphylococci causing IMI. Preliminary results for 12 of the 30 isolates show 100% agreement between *blaZ* carriage and phenotypic susceptibility to penicillin (using the agar dilution method, Table 1). All eight isolates negative for *blaZ* based on whole genome sequencing were susceptible to penicillin *in vitro,* while the four *blaZ-*positive isolates were determined to be resistant. Although no other antimicrobial resistance determinants were identified from genotypic data for these isolates, we may potentially see phenotypic resistance to other antimicrobial compounds when they are tested *in vitro.* Databases identifying antimicrobial resistance are only looking for previously-described genes and mechanisms of resistance in bacteria; the process of identifying novel mechanisms of resistance are begin by observing phenotypic resistance in the absence of previously-described resistance determinants.

Resistance determinants in bacteria can be carried either as part of a genetic mobile element (usually a plasmid) or chromosomally. Location of *blaZ* carriage is not well characterized for *Staph. chromogenes*, but a study of *S. aureus* IMI isolates in Finland and Norway found that 26 out of 34 Finnish isolates (76.5%) and 25 out of 44 Swedish isolates (56.8%) carried *blaZ* on a plasmid (vs. chromosomally) (Bagcigil et al., 2012). Extrapolating from these results, *blaZ* could potentially be carried either chromosomally or on a plasmid for the *Staph. chromogenes* isolates in the current study. Preliminary work exploring location of *blaZ* gene for the 10 positive isolates in the current study found that four isolates were negative for plasmid carriage (using Plasmid Finder from the Center for Genomic Epidemiology, Technical University of Denmark, <https://cge.food.dtu.dk/services/PlasmidFinder/>). One of these four isolates was from ST6, one was from a different farm and belonged to ST51, and 2 from a third farm both belonged to ST5. As *blaZ* carriage appeared to be primarily a function of ST in Chapter 4, and consistent carriage of *blaZ* from ST originating from different farms is observed, this may suggest that *blaZ* is primarily located chromosomally for this population of *Staph. chromogenes* isolates. The only ST identified from Chapter 4 which did not have consistent carriage of *blaZ* was ST6. It may be that these isolates of ST6 carry *blaZ* on a plasmid. If resistance genes are harbored on horizontally transmissible elements (such as plasmids), strains carrying these elements can successfully disseminate them to new, previously-susceptible bacteria, belonging to both the same species and different species. Location of resistance determinants on plasmids generally promotes more efficient spread of resistance gene (Malachowa and DeLeo, 2010). Studies exploring whether *blaZ* is more likely to be carried chromosomally or on a plasmid for *Staph. chromogenes* from bovine IMI, would be useful in understanding transmission of penicillin resistance for this predominant mastitis pathogen.

*6.2.3 Further work exploring the intraspecies diversity of Staph. chromogenes*

Among the 30 *Staph. chromogenes* strain-typed using MLST in Chapter 4, there were 10 different ST identified. As the MLST scheme for *Staph. chromogenes* was described fairly recently (Huebner et al., 2021), the number of studies describing strain-typing results using this scheme to date is limited (Petzer et al., 2022; Persson Waller et al., 2023). Describing the diversity of *Staph. chromogenes* using MLST is a rapidly growing area of research. Four of the 10 ST in the current study had previously not been described, and 43% of all isolates belonging to 33 new ST were identified by Persson Waller et al. (2023). These results highlight the importance of contributing to publicly-available databases in order to improve our ability to better understand the diversity of this common mastitis pathogen. Some overlapping ST (those related to ST1 and ST6) were observed between the Swedish isolates in Persson Waller et al. (2023), the Vermont isolates in the current study, and the isolates from Washington State, Vermont, and Belgium described in Huebner et al. (2021). However, distribution of different *Staph. chromogenes* ST also seems to be a function of geographical location. ST15 was the second most commonly identified by Hubener et al. (2021) after ST1, with 17/120 (14.2%) of isolates belonging to this ST. In their study, ST15 was primarily identified in isolates from Vermont and Washington State (16/17 isolates), and less frequently in Belgium. Interestingly, although all isolates in the current study are from Vermont, only 2 of 30 isolates belonged to ST15. A study using MLST to strain-type larger number of *Staph. chromogenes* associated with bovine IMI from numerous countries across multiple continents would help us to better understand how the distribution and diversity of this pathogen varies with geography.

In the longitudinal study described in Chapters 3-5, MALD-TOF mass spectrometry was used to identify bacterial isolates to species level. Isolates were sent to the University of Missouri to be run on the MALDI-TOF, where 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, which has been specifically informed to better identify isolates important to bovine mastitis. In total, 488 bacterial isolates were identified by the MALDI-TOF as *Staph. chromogenes.* Three isolates later identified to be *Staph. chromogenes* (using sequencing of the *tuf* gene) were called “no ID” by MALD-TOF. These three isolates are all from the same farm, and two were isolated from the same quarter over sequential visits. It is interesting to consider why these three particular *Staph. chromogenes* isolates out almost 500 were unable to be identified with MALDI-TOF. Unfortunately, in the current project none of these isolates were strain-typed using RAPD or MLST, so whether or not they are the same ST is unknown. However, it is tempting to wonder if they belong to a certain ST which is different enough from the ST within *Staph. chromogenes* that they were unable to be identified in the same manner. Further, if this was the case, it may lead to the question of whether MALDI-TOF could ever be used for strain-typing isolates of *Staph. chromogenes.* If so, it would be significantly easier and faster than any current method of strain-typing for this organism. In order to explore this question, one would need a large set of *Staph. chromogenes* isolates, with a diversity of MLST identified and represented, and a substantial number of isolates belonging to each ST.

Although we did not find any support for an association between carriage of *blaZ* and the associated SCC category of an IMI, results from Chapter 4 suggest that *blaZ* carriage is likely a function of ST in *Staph. chromogenes*. As mentioned above, for all but 1 of the 5 MLST identified, *blaZ* carriage was uniform across a ST. Similar to *blaZ*, the carriage of virulence factors by isolates in the current study also appears to be a function of ST. The cumulative number of virulence factors identified belonging to the 5 ST with multiple isolates showed little to no variation, and many of the limited differences in presence or absence of virulence factors occurred at the ST level. Association between ST and carriage of methicillin resistance has been demonstrated for bovine *Staph. aureus.* Evidence for association between ST and virulence potential, as well as ST and carriage antimicrobial resistance determinants, was observed in Persson Waller et al. (2023) for *Staph. chromogenes.* When Naushad et al. (2019) applied various clustering approaches in order to determine whether particular VF distributions had any association with SCC category or clinical mastitis, NASM isolates instead grouped together by their respective species. Resistance profiles for NASM are species-specific, and it also appears that the pattern of virulence genes carried by NASM isolates is also likely species-dependent. Therefore, a genetic basis for carriage of virulence factors or antimicrobial resistance determinants may also extend to the strain level.Our ability to explore if pattern and number of virulence factors or carriage of *blaZ* varies by ST in the current study was limited, both by the relatively small number of isolates assigned to most ST identified, as well as complete uniformity in the number and type of virulence factors and *blaZ* presence for a particular ST. Future studies with larger isolate collections, isolates collected from a greater number of farms, and the ability to sequence a larger number of *Staph. chromogenes* isolates may be better able to explore associations of antimicrobial resistance determinants and virulence factor carriage by ST (as determined by MLST), as larger sample sizes would likely result in a greater diversity of ST and a greater ability to account for the effect of clustering by herd.

Although no evidence was found that the number of virulence factors or antimicrobial resistance genes would predict if a *Staph. chromogenes* isolate would be associated with a persistent IMI with a high or low SCC, it may be interesting to compare virulence profiles and antimicrobial resistance gene carriage between isolates associated with clinical vs. non clinical mastitis. In a study of 217 S. aureus IMI isolates typed using pulsed-field gel electrophoresis (PFGE) Haveri et al. (2005) identified a particular pulsotype which was significantly associated with severe clinical mastitis symptoms but reduced persistence when compared to the 4 other commonly identified pulsotypes. This association between a specific clinical traits associated with an IMI (persistency and clinical severity) has not yet been widely described for *Staph. chromogenes*, but may help us better understand if particular pathotypes or ST exist in the population which are of greater concern when causing IMI.

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| **Table 6.1** Agreement between *blaZ* gene carriage and phenotypic susceptibility (using agar dilution) to penicillin by strain type (ST; as determined by MLST) for 30 *Staphylococcus chromogenes* isolates associated with persistent bovine intramammary infections. Isolates were defined as susceptible to penicillin if their MIC was 0.12 µg/mL or lower, and resistant if their MIC was 0.25 µg/mL (2018 Clinical & Laboratory Standards Institute guidelines). N = no *blaZ* carriage; Y = positive for *blaZ* carriage; S = susceptible to penicillin; R = resistant to penicillin. | | | | |
| Isolate | ST | ST cluster | *blaZ* carriage | Susceptibility phenotype (penicillin) |
| assembly\_3 | 175 | 1 | **N** | S |
| assembly\_2 | 174 | 1 | **N** | S |
| assembly\_20 | 176 | 1 | **N** | - |
| assembly\_21 | 176 | 1 | **N** | - |
| assembly\_22 | 176 | 1 | **N** | - |
| assembly\_23 | 176 | 1 | **N** | - |
| assembly\_14 | 176 | 1 | **N** | - |
| assembly\_15 | 176 | 1 | **N** | - |
| assembly\_16 | 176 | 1 | **N** | - |
| assembly\_17 | 176 | 1 | **N** | - |
| assembly\_19 | 176 | 1 | **N** | - |
| assembly\_10 | 5 | 5 | **Y** | - |
| assembly\_24 | 5 | 5 | **Y** | - |
| assembly\_12 | 5 | 5 | **Y** | R |
| assembly\_1 | 6 | 6 | **N** | S |
| assembly\_6 | 6 | 6 | **N** | S |
| assembly\_9 | 6 | 6 | **N** | S |
| assembly\_13 | 6 | 6 | **N** | S |
| assembly\_30 | 6 | 6 | **N** | - |
| assembly\_25 | 6 | 6 | **Y** | - |
| assembly\_26 | 6 | 6 | **Y** | - |
| assembly\_27 | 6 | 6 | **Y** | - |
| assembly\_29 | 6 | 6 | **Y** | - |
| assembly\_5 | 25 | 25 | **N** | S |
| assembly\_7 | 25 | 25 | **N** | S |
| assembly\_4 | 48 | 48 | **Y** | R |
| assembly\_11 | 48 | 48 | **Y** | R |
| assembly\_8 | 51 | 51 | **Y** | R |
| assembly\_28 | 136 | 136 | **N** | - |
| assembly\_18 | 177 | 177 | **N** | - |

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