Udder health on organic dairy farms in Vermont: a focus on the epidemiology of staphylococci causing intramammary infections in dairy cattle

A Dissertation Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

Specializing in Animal, Nutrition, and Food Sciences

October, 2024

Defense Date: August 26, 2024

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ABSTRACT

While all dairy producers rely on best management practices to support cow health, mastitis control is of the utmost importance in the prevention of intramammary infections (IMI) on organic farms. Understanding the epidemiology of mastitis pathogens leads to more effective measures which prevent or limit transmission of IMI. This work sought to better understand the epidemiology of the most relevant pathogens causing IMI in organic dairy cows in Vermont.

An observational study of organic dairy herds was undertaken to identify differences in udder health outcomes between herds using different housing systems. 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. Species from the genus *Staphylococcus* were responsible for the majority of mastitis in these herds. *S. 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 no growth 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 *S. chromogenes IMI*, with some causing an elevation comparable to that of major mastitis pathogens and others similar to no growth quarters. The majority of *S. chromogenes* IMI were persistent. A study to better understand the epidemiology of this subgroup was completed, with two categories selected from persistent *S. chromogenes* IMI: 15 associated with high SCC and 15 with low SCC. Representative bacterial isolates from all 30 IMI were submitted for whole genome sequencing and strain-typed. Particular strain types of *S. 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 (10 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 appeared to be a function of strain type.

This work generated foundational knowledge about staphylococci causing mastitis on organic dairy farms in Vermont. Mitigating the effect of mastitis caused by staphylococci through targeted prevention and control measures helps dairy producers achieve quality price premiums and results in a higher-quality product for consumers.

CITATIONS

Material from this dissertation has been published in the following form:

Jeffrey, C.E., Andrews, T., Godden, S.M., Neher, D.A., Barlow, J.W.. (2024). Relationship Between Facility Type and Bulk Tank Milk Bacteriology, Udder Health, Udder Hygiene, and Milk Production on Vermont Organic Dairy Farms. Journal of Dairy Science. Epub ahead of print. DOI: 10.3168/jds.2023-24576. PMID: 38908690.

AND

Material from this dissertation has been submitted for publication to the Journal of Dairy Science on August 14th, 2024 in the following form:

Jeffrey, C.E., Adkins, P.R.F., Dufour, S., Barlow, J.W.. Staphylococci and mammaliicocci: which species are important for udder health on organic dairy farms? Journal of Dairy Science.

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CHAPTER 1: Literature review – Antimicrobial susceptibility of bovine staphylococcal mastitis isolates on organic vs. conventional dairy farms

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1.1 Abstract

An unfortunate consequence of any antimicrobial use is the potential to select for the emergence of resistant strains of bacteria in a population. A unique opportunity in which to assess the effect of antimicrobial use on resistance of mastitis pathogens is to compare dairy farms which are managed “conventionally” to those that are managed “organically.” Without the selective pressure of antimicrobial usage (as on organic dairies), it would be expected that resistant bacterial strains would gradually be replaced by susceptible strains if an advantage was no longer conferred by carriage of antimicrobial resistance (AMR) genes. The objective of this narrative review was to summarize studies which compared the relationship between antimicrobial usage at the farm level (organic vs. conventional) and AMR of bovine staphylococcal mastitis isolates, the predominant group of bacteria causing intramammary infections in dairy cattle globally. Other potential explanatory factors for differing antimicrobial susceptibility of staphylococci causing intramammary infections are also described. These include differences in AMR carriage between staphylococcal species and various risk factors associated with the prevalence of different species causing intramammary infections in a particular herd. Overall, studies comparing AMR of mastitis-associated staphylococci between herds under organic management and herds managed conventionally find either no difference or that isolates originating from organic farms exhibit slightly more susceptibility. Although some level of resistance was observed against a number of antimicrobials important for veterinary medicine (cephalosporins, penicillin, tetracycline), overall resistance of mastitis-associated staphylococci is generally low and the most commonly-used mastitis treatments are still effective. Studies exploring this issue varied widely in their approach, including use of differing methodology to determine susceptibility patterns and variation in sampling scheme. Most studies were carried out in either the US or Europe. This is somewhat problematic, as definitions of “organic” differ for dairies in the EU (where antimicrobial usage is still allowed, but is more tightly regulated and limited) and the US (any animal treated with antimicrobials must leave the herd). However, the overall conclusions from studies comparing the two different management systems are still informative. Directions for future work could include comparing AMR for staphylococci between these two systems while controlling for species, comparison of predominant strain types within a given species between organic and conventional farms, or long-term studies of farms transitioning from conventional to organic status to better understand what types of AMR are maintained in organic dairy herds and for how long.

1.2 Introduction

Effective antimicrobial therapy is a cornerstone of livestock veterinary medicine, maintaining the health of animals producing food and fiber to support the global population and alleviating suffering due to infectious disease. However, use of antimicrobial agents is inherently a “powerful selective force that promotes the emergence of resistant strains,” and the cumulative effect of antibiotic use in general has “clearly been to increase the prevalence of resistance in the population [of bacteria] as a whole” (Lipsitch and Samore, 2002). Resistance to antimicrobials can be acquired by bacteria in multiple ways. Spontaneously occurring genetic mutations (passed vertically to daughter cells) can confer antimicrobial resistance, but more commonly it is acquired by the horizontal transfer of mobile DNA elements from a donor cell, often another species of bacteria (Chambers, 2001; Sefton, 2002). In the case of horizontal transfer, antimicrobial resistance genes can become rapidly and widely disseminated throughout a bacterial population. This occurs either by further genetic exchanges between the newly-resistant strain and susceptible strains, or by clonal spread of the newly-resistant strain itself (Chambers, 2001). Although the interplay between development of resistance and antimicrobial use is complex and multifactorial, it is generally accepted that antimicrobial resistance (AMR) is potentially amplified in both human healthcare environments and on farms, where frequent exposure to antimicrobial compounds can select for resistant populations of bacteria (Parker et al., 2024). A direct temporal relationship between antimicrobial use and resistance has been described, both in human healthcare settings over the long-term (López-Lozano et al., 2000) and in transient increases in resistant fecal bacteria in cattle (Stabler et al., 1982; Langford et al., 2003; Berge et al., 2005; Lowrance et al., 2007). It has been suggested that antimicrobial usage in food animals could negatively affect human health by influencing the selection of drug-resistant foodborne pathogens (Yan and Gilbert, 2004). However, the risk of transmission of resistant bacteria between farm systems and humans is not fully understood; selection for resistant bacteria and transfer of AMR genes occurs through a variety of mechanisms, and is not always linked to use of a specific antibiotic (Mathew et al., 2007).

The most “obvious selection pressure for AMR” on cattle farms is the use of antimicrobials for treating sick animals (Call et al., 2008). Specifically, this can promote AMR on cattle farms by two potential mechanisms: 1) treatment with antimicrobials provides a competitive advantage for strains that carry resistance to that particular drug, allowing the relative proportion of resistant bacteria in a populations to increase; and 2) if resistance genes are harbored on horizontally transmissible elements (plasmids or conjugative transposons), strains carrying these elements can then successfully disseminate them to new, previously-susceptible bacteria (Call et al., 2008). The primary reason for antimicrobial drug usage in adult dairy cows in the US is for treatment of mastitis (Pol and Ruegg, 2007b). Bacteria belonging to the genus *Staphylococcus*, which broadly includes the major mastitis pathogen *Staphylococcus aureus* and a heterogeneous group of bacteria known as the non-*aureus* staphylococci and mammaliicocci (NASM), are the predominant pathogens causing intramammary infections (IMI) in dairy animals worldwide (as summarized in De Buck et al., 2021). A limited number of antimicrobials are approved for treatment of mastitis in lactating dairy cattle in the US, including various β-lactams (penicillin, cephapirin, ceftiofur, amoxicillin, hetacillin, and cloxacillin) and one lincosamide (pirlimycin) (FARM, 2020). At this time, *S. aureus*, NASM, and other mastitis pathogens are generally susceptible to the antibiotics currently used to treat IMI (Kolar et al., 2024; Pol and Ruegg, 2007b; with the notable exception of some studies finding *S. aureus* and NASM exhibiting moderate resistance against penicillin, see below). However, efforts to continue surveying and understanding the AMR patterns for these ubiquitous mastitis pathogens is warranted. The importance of *S. aureus* as a human pathogen is well-established (Tong et al., 2015), and virulence genes known to cause disease in both humans and animals have been demonstrated in NASM isolates from bovine IMI (Park et al., 2011; Unal and Cinar, 2012). Additionally, transmission of resistance genes between different staphylococcal species have led to the idea that NASM may act as a “reservoir” of AMR for more pathogenic staphylococcal species such as *S. aureus* (Cuny et al., 2017; Feßler et al., 2018; Khazandi et al., 2018).

A unique opportunity in which to assess the effect of antimicrobial use on AMR of these important mastitis pathogens is to compare dairy farm systems which are managed “conventionally” to those that are managed “organically.” Although the definition can differ by region (namely, the US and EU; see below), antimicrobial usage on “organic” dairies is usually less or non-existent when compared to “conventional’” dairy farms. When comparing bacterial isolates of bovine origin from these two types of systems, the general hypothesis is that AMR would be expected to diminish in prevalence when antimicrobial use is decreased or discontinued. Without the selective pressure of antimicrobial usage (as on organic dairies), bacterial strains containing resistance genes would gradually be replaced by susceptible strains, as selective advantage is no longer conferred by AMR carriage (assuming AMR carriage incurs a fitness cost; see below). The goal of this narrative review is to summarize studies which compared the relationship between antimicrobial usage at the farm level (organic vs. conventional) and antimicrobial susceptibility of bovine staphylococcal mastitis isolates.

1.3 Limitations and caveats for comparisons between studies

An important qualification when considering the body of work comparing resistance patterns of mastitis pathogens between management systems is that “organic” dairies differ between the US and Europe, where the majority of these studies have been carried out. Organic regulations in European countries still allow for some antimicrobial use (albeit with extended withdrawal periods and stricter veterinary oversight; EU Commission, 2024), while organic regulations in the US mandate that any animal treated with antimicrobials be permanently removed from the herd (USDA, 2024). The level of on-farm antimicrobial usage (and therefore selective pressure for resistance) therefore differs between European and US dairies, making comparisons between studies carried out under these varying regulations somewhat complicated. Specific rules for both organic dairy production certifications have evolved over time (Dimitri and Nehring, 2022; Grodkowski et al., 2023), further adding to the nuance of what is meant by “organic” dairy production in a retrospective analysis. The specific antimicrobials approved for usage in livestock varies by country, as well as which compounds are most commonly-used (e.g., for mastitis: penicillin in Finland, Taponen 2023; cephalosporins in the US, de Campos 2021). Even within the US, the amount and type of antimicrobials used in dairy cows changes over time as new products are developed or regulations around usage shift (USDA, 2009). Consequently, geographic and temporal differences can affect the type and amount of antimicrobial selective pressure experienced by mastitis pathogens on dairy farms.

Direct comparison of antimicrobial sensitivity results across studies can be problematic for a number of reasons. Importantly, the methodology used to determine the minimum inhibitory concentration (MIC) or categorization of an isolate as susceptible or resistant varies between studies. Further, 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 isolates of *S. aureus* associated with bovine mastitis, both of these types of discrepancies have been reported for penicillin resistance (Sampimon, 2009; Taponen et al., 2023). This also holds true for the other staphylococci; as summarized by Sampimon (2009), “agreement between phenotypic and genotypic test results for assessment of resistance of CNS of bovine origin to penicillin, oxacillin, and ML [macrolide] antibiotics depended on the antimicrobial compound of interest and on methods used to analyse and interpret test results, but was rarely perfect.” 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. Disagreements have also been described within different methods of phenotypic determination of resistance for mastitis pathogens. A study comparing commercially-available broth microdilution plates (Sensititre Custom Plates) and agar disk diffusion for determining antimicrobial susceptibility of bovine IMI isolates found fair agreement overall (80.7%) between the two methods, but this varied based on the particular bacterial-antimicrobial combination tested (Palladini et al., 2023). No NASM species were included, but there was satisfactory agreement (89 to 100%) for *S. aureus* and all antimicrobial agents tested. In a study comparing Sensititre (broth microdilution) and disk diffusion for determining AMR in clinical mastitis pathogens, agreement was good for most isolate-antimicrobial MIC combinations (Saini et al., 2011). An important exception to this was that diagnostic accuracy was low when *S. aureus* was tested against both ceftiofur and oxacillin using either method. Low correlation was also found when *S. aureus* was tested against erythromycin and neomycin in another study comparing 2 dilution methods to determine MIC and disk diffusion diameters for mastitis-associated isolates (Klement et al., 2005). Further complicating comparison of AMR profiles between studies is shifting criteria for classifying an isolate as susceptible or resistant. Breakpoints for antimicrobial susceptibility testing are updated every few years, and multiple conflicting standards exist for categorization of resistant or susceptible bacteria which are dependent on geographical location (Clinical & Laboratory Standards Institute, CLSI; European Committee on Antimicrobial Susceptibility Testing, EUCAST).

Difference in sampling scheme for studies collecting milk from individual cows will affect observed prevalence of resistance in bacteria isolated from samples. Within the studies summarized in this review, sampling strategies for quartermilk and criteria for cow inclusion vary widely. Some studies included sampled cows in a herd at random or without using any specific criteria (Tikofsky et al., 2003; Bombyk et al., 2008; Garmo et al., 2010), while others used the California Mastitis test (CMT) to selectively sample cows with evidence of extant mastitis (Busato et al., 2000; Roesch et al., 2006). Bennedsgard et al. (2006) used a specific set of criteria in order to maximize their chances of sampling cows with *S. aureus* IMI specifically, while others sampled only multiparous cows in the herd (Pol and Ruegg, 2007a; McDougall et al., 2021). Sampling multiparous cows exclusively increases the likelihood samples collected will have an IMI, as increasing parity is a risk factor for mastitis generally (Barkema et al., 1998; Busato et al., 2000) and IMI with *S. aureus* specifically (Zadoks et al., 2001; Tenhagen et al., 2006). The likelihood of different NASM species causing IMI varies by parity, and resistance patterns are species-specific for NASM (see below). Therefore, sampling multiparous cows exclusively will bias which species are included and thereby the resistance profiles of mastitis pathogens described. A further consideration is whether the bacteria included were associated with cases of subclinical mastitis, clinical mastitis, or both. AMR has been shown to be more prevalent in NASM isolates associated with clinical vs. subclinical mastitis, so inclusion criteria around sample type will affect the observed AMR prevalence. Oxacillin resistance was more frequent in clinical mastitis isolates (56.5%) vs. subclinical mastitis isolates (43.9%; Frey et al., 2013), β-lactamase production was more common in subclinical vs. clinical cases (Persson Waller et al., 2011), and Wuytack et al. (2020) found carriage of the resistance gene *mecA* was proportionately higher in NASM isolates causing clinical vs. subclinical infection. However, as certain NASM are more likely to be associated with clinical mastitis vs. subclinical mastitis and vice versa (Persson Waller et al., 2011; although, see Condas et al., 2017b) and resistance patterns of NASM are species-specific (see below), this observed difference in AMR prevalence between sample type may ultimately result from species differences between the 2 categories. In Persson Waller et al. (2011), *S. epidermidis* and *S. saprophyticus* were more prevalent in subclinical vs. clinical mastitis, while *S. hyicus* was more common in clinical mastitis. The authors attribute the higher proportion of penicillin resistance in subclinical isolates to the high prevalence of *S. epidermidis* and *S. saprophyticus* in these samples, as these species demonstrated significantly more penicillin resistance when compared with other NASM. Further support that differences in AMR for NASM associated with clinical vs. subclinical mastitis is primarily a result of species differences is found in Naushad et al. (2018). In their analyses of 328 NASM isolates from samples with subclinical mastitis and 57 isolates from clinical mastitis, within the same species, no significant differences existed in the prevalence of drug-specific AMR or resistance determinants when contrasting the two sample types.

1.4 Summary of studies describing AMR of staphylococci from conventional vs. organic dairies

Nomenclature for the group of staphylococci causing bovine IMI excluding *S. aureus* has shifted over the past few decades, as both phylogeny and techniques for species-level identification have evolved. Some species which had been previously identified as staphylococci were recognized more recently as belonging instead to a closely related genus (*Mammaliicoccus*), and identification methods beyond a coagulase test have become more widely used. Although NASM is used throughout the rest of the review, the terminology used below when referring to results of a specific study is consistent with authors’ language and groupings of organisms (e.g., “coagulase-negative staphylococci,” or “CNS;” “non-*aureus* staphylococci,” or “NAS”). This decision was made in an attempt to be consistent with the original authors’ contemporary understanding of phylogeny and methodology.

Overall, studies comparing AMR of mastitis-associated staphylococci between herds under organic management and herds managed conventionally find either no difference or that isolates originating from organic farms exhibit slightly more susceptibility (Table 1.1). However, these studies vary widely in their approach to exploring this question, primarily in number of isolates included and herds sampled, as well as approach to statistical analysis. In a descriptive study from Switzerland, Busato et al. (2000) found that the proportions of *S. aureus* isolates from organic herds (ORG) resistant to different antimicrobials were equivalent to those from conventional herds (CON). Similarly, the proportions of resistant isolates of CNS were comparable between the two systems, with the exception of a numerically higher proportion resistant to rifamyin from organic herds. A limitation of this study is that the data describing susceptibility of staphylococci from conventional herds was from a previously unpublished survey by the authors, and not contemporaneous with analysis of the organic isolates. In another descriptive study, researchers in Norway (Garmo et al., 2010) found similar proportions of *S. aureus* and CNS isolates resistant to penicillin between the two herd types (*S. aureus*: 6/68 or 8.8% from CON, vs. 9/64 or 14.0% from ORG; CNS: 81/167 or 48.5% for CON, vs. 93/200 or 46.5% from ORG). The authors note that penicillin resistance was proportionately higher in CNS vs. *S. aureus* isolates, consistent with more recent work looking at the resistance of staphylococci from bovine milk samples (as summarized in Taponen et al., 2023). In a Swiss study comparing resistance profiles of NAS and *S. aureus* from quartermilk samples, Roesch et al. (2006) also found that NAS isolates exhibited a higher overall percentage of AMR than *S. aureus* isolates. For 12 antimicrobials representing either drugs used to treat mastitis in dairy herds or drugs important in human medicine, they found that percentage of AMR did not differ significantly between *S. aureus* and NAS isolates from cows kept on organic vs. conventional herds. Although the overall proportion of *S. aureus* isolates resistant to ≤1 antimicrobial was numerically higher from organic cows (16/46, 35%) vs. conventional cows (6/33, 18%), this difference was not statistically significant. The proportion of NAS isolates resistant ≤1 antimicrobial to between systems was very similar (ORG: 9/19, 47%; CON: 10/19, 53%).

In contrast, Bombyk et al. (2008) found that staphylococci causing mastitis on organic dairies were associated with more overall antimicrobial susceptibility than those from conventional farms. For this study, researchers differentiated mastitis-associated staphylococci into 3 categories: coagulase-positive *Staph*. (CPS), novobiocin-sensitive CNS (NSCNS), and novobiocin-resistant CNS (NRCNS). In an analysis combining all 3 groupings of staphylococci, a larger proportion of isolates from organic herds were susceptible to pirlimycin and tetracycline compared with those from conventional herds. Susceptibility to erythromycin and penicillin did not differ significantly by herd type when all staphylococci were combined (CON vs. ORG). No significant differences between organic and conventional systems were found for *S. aureus*, although the numbers of isolates found was fairly small compared to both categories of CNS (36 *S. aureus* vs. 210 NSCNS and 159 NRCNS). When each category of CNS (novobiocin-susceptible or resistant) was analyzed separately, isolates within both groups from organic herds were more likely to be susceptible to pirlimycin than CNS from conventional dairies. No difference in tetracycline, erythromycin or penicillin susceptibility was seen between herd types (CON vs. ORG) within either CNS category. A larger proportion of NSCNS vs. NRCNS (when analyzed separately for conventional and organic herds) were susceptible to tetracycline, leading the authors to suggest that management practices unrelated to antimicrobial use may contribute to the observed differences in susceptibility patterns of CNS on dairy herds.

A number of studies comparing resistance patterns of mastitis-associated bacteria between conventional and organic dairy systems have focused specifically on *S. aureus*. Researchers in New York and Vermont (US) found that *S. aureus* isolates from both types of herds showed good susceptibility to most antimicrobials used to treat mastitis, but isolates from organic herds were significantly more susceptible (Tikofsky et al., 2003). In this study, researchers took two different approaches to analyzing the data: 1) the strength of association between the proportion of susceptible and resistant isolates was evaluated by management category, and 2) numeric differences in mean zone diameter were compared for isolates from organic vs. conventional herds. When results were combined over both analyses, *S. aureus* isolates from organic herds were more susceptible than those from conventional herds for 7 of the 9 antimicrobials studied. Contrary to these findings, researchers comparing resistance of isolates from bulk tank milk of organic and conventional systems in both the US and Denmark found that overall, antimicrobial susceptibility was very similar for *S. aureus* in both countries (Sato et al., 2004). Bulk tank isolates from conventional herds in Wisconsin (US) had significantly reduced susceptibility to ciprofloxacin (vs. isolates from organic herds), and isolates from organic herds in Denmark had reduced susceptibility to avilamycin (vs. isolates from conventional herds). In a finding highlighting the importance of geography in epidemiological studies, authors point out that differences in the antimicrobial susceptibility of *S. aureus* isolates between organic and conventional herds were small relative to differences in resistance patterns observed between countries. In agreement with Sato et. al, Bennedsgaard et al. (2006) observed no statistically significant differences in the prevalence of cows with penicillin-resistant *S. aureus* mastitis or the proportion of *S. aureus* isolates from quartermilk resistant to penicillin between conventional and organic dairies in Denmark.

Two studies looking at bulk tank milk (BTM) focused on detection of staphylococci carrying genetic determinants conferring penicillin resistance (*mecA* and *mecC* genes), an important consideration for public health globally. In a large study with the goal of surveilling dairy-associated methicillin-resistant *S. aureus* (MRSA) in Germany, researchers collected BTM from 372 conventional and 303 organic herds (Tenhagen et al., 2018). Using binary logistic regression to describe association of MRSA-positive samples with herd type (conventional vs. organic), they found that the prevalence of MRSA was significantly higher in BTM samples from conventional herds (9.7%) compared with organic herds (1.7%). The model-based approach allowed researchers to control for the effects of geographical region and herd size, both of which were also significant predictors of MRSA herd status. When comparing the proportion of BTM MRSA isolates resistant to 12 different antimicrobials between conventional and organic herds, MRSA isolates from conventional farms tended to be more resistant. However, as there were a limited number of isolates from organic herds (n = 5) compared to conventional herds (n = 36), no statistical analyses were performed. A large, multistate study in the US sampled BTM from 192 organic herds and 100 conventional herds matched for geographical location and herd size (Cicconi-Hogan et al., 2014). They identified 13 isolates from BTM as methicillin resistant (*mecA*-positive): 7 isolates from conventional herds and 6 from organic. Using 16S rRNA and *rpoB* genes for species-level identification, these 13 isolates were identified as *S. aureus* (n = 1), *S. sciuri* (n = 5), *S. chromogenes* (n = 2), *S. saprophyticus* (n = 3), *S. agnetis* (n = 1), and *Macrococcus caseolyticus* (a genus closely related to staphylococci; n = 1). Surprisingly, the single methicillin-resistant *S. aureus* isolate was from an organic herd, for an observed 0.3% prevalence of MRSA at the herd level. Methicillin-resistant CNS were found at a prevalence of 2% in the organic population and 5% in the conventional population. The authors highlight the relatively large number of methicillin-resistant *S. sciuri* identified (6 out of the 12 methicillin-resistant CNS) compared with previous work, and also suggest that a potential methicillin-resistant *Staphylococcus* reservoir in the dairy herd population of the US may be independent of the type of production system. To this point, Walther and Perreten (2007) report the occurrence of a dairy cow on an organic farm in Switzerland that was diagnosed twice within 2 months with subclinical mastitis caused by methicillin-resistant *S. epidermidis*. The two strains had identical PFGE patterns of chromosomal DNA, exhibited resistance to chloramphenicol, and contained streptomycin- and trimethoprim-resistance genes but did not display phenotypic resistance against these drugs in vitro. Furthermore, the second *S. epidermidis* isolate contained an additional aminoglycoside-resistance gene, indicating the potential acquisition of resistance by horizontal gene transfer since isolation of the first bacterium. Similar to Cicconi-Hogan et al. (2014), the authors highlight that this finding demonstrates cows on organic farms may harbor multidrug-resistant staphylococci despite the limited use of antimicrobials under EU organic regulations.

Perhaps a limitation of the above studies comparing the resistance of staphylococci from organic and conventional dairy farms is that limited or no quantification of on-farm antimicrobial usage was calculated or presented. In order to evaluate if the level of antimicrobial usage in food animals selects for drug-resistant pathogens, an important component in a study exploring this question would be a quantification of antimicrobial use at the farm or cow level to be able to estimate the amount of selective pressure exerted on intramammary pathogens. Although all antimicrobial usage is prohibited on US organic dairies, the amount and type of antimicrobials used by conventionally-managed farms can vary widely (Pol and Ruegg, 2007b). Two of the largest-scale, statistically robust studies comparing the resistance profiles of staphylococci from quartermilk samples between conventional and organic dairies include a detailed, numeric quantification of antimicrobial usage by enrolled farms. In a 2007 study in the US, Pol and Ruegg report a standardized level of exposure to 10 different antimicrobials by calculating of the number of defined daily doses used per cow on each enrolled farm, and then categorize the 40 enrolled herds based on their respective antimicrobial usage. Herds are categorized into 3 groups: organic (no antimicrobial usage), conventional–low usage (conventional farms not using or using ≤ the first quartile of use for each drug; CON-LO), and conventional–high usage (conventional farms using > the first quartile for a particular drug; CON-HI). The authors took multiple approaches to compare resistance among isolates from the 3 antimicrobial usage groups. First, they compared the proportion of each type of isolate (CNS or *S. aureus*) that was susceptible or resistant in each category (CON vs. ORG) using a categorical test of association, in order to explore if proportion of susceptible isolates was independent of herd type. Secondly, they used a test of association to explore if the MIC for each type of isolate (CNS or *S. aureus*) was independent of herd type (CON vs. ORG). Lastly, they performed survival analysis for each type of isolate (CNS or *S. aureus*) based on the 3 antimicrobial usage categories (ORG, CON-LO, or CON-HI). In this last analysis of “time to event,” antimicrobial concentration in wells of the susceptibility test was considered “time,” and the “event” was inhibition of any bacterial growth. Overall, Pol and Ruegg found that isolates from organic herds were more susceptible to antimicrobials than those from conventional herds. Specifically, for *S. aureus*: (1) isolates from conventional herds were more likely to be resistant to ampicillin and penicillin when compared with isolates from organic herds, and herd type was not associated with the proportion of resistant isolates for the other antimicrobial drugs tested; (2) isolates from conventional herds had a higher MIC for pirlimycin and sulfadimethoxine compared with isolates from organic herds, and herd type was not associated with the MIC of the other antimicrobial drugs tested; and (3) in the survival analysis, the MIC that inhibited 90% (MIC90) of *S. aureus* isolates from organic herds for penicillin and pirlimycin was lower than the MIC90 of the isolates from CON-LO and CON-HI herds (MIC50, the MIC that inhibited 50% of isolates, was not different for these drugs). For CNS: (1) isolates from conventional herds were more likely to be resistant to ampicillin, penicillin, pirlimycin, and tetracycline compared with isolates from ORG herds, and herd type was not associated with the proportion of resistant isolates for the other antimicrobial drugs tested; (2) isolates from conventional herds had a higher MIC for ampicillin, pirlimycin, and tetracycline compared with isolates from organic herds, and herd type was not associated with the MIC of the other antimicrobial drugs tested; and (3) in the survival analysis, the MIC90 of CNS isolates from organic herds for ampicillin, penicillin, pirlimycin, and tetracycline was lower than the MIC90 of the isolates from CON-LO and CON-HI herds (ORG and CON-LO herds had a lower MIC50 for erythromycin than CON-HI herds, but the MIC90 did not differ by usage group). The authors highlight that although some differences were found between antimicrobial usage groups, most isolates from all farm types were inhibited at the lowest dilution tested of most antimicrobial drugs routinely used on dairy farms.

The other study comparing resistance of staphylococci between organic and conventional dairies to include a detailed quantification of antimicrobial usage enrolled 7 organic herds, 11 conventional herds using ampicillin-cloxacillin dry cow therapy (CON-AC), and 8 conventional herds using cephalonium dry cow therapy (CON-CE) in New Zealand (McDougall et al., 2021). Although the study was carried out in NZ, participating herds were all certified under the USDA National Organic Program. Conventional herds of both categories were selected on the basis that >50% of the cows were treated in each of the 3 previous years with at least 1 dry cow therapy (DCT) product. Similar to Pol and Ruegg (2007a), the authors took a multifaced approach to exploring the resistance patterns of *S. aureus* and CNS from organic and conventional systems. Overall, the MIC of CNS from ORG herds were lower than isolates from both types of CON herd. For *S. aureus*, they found that the MIC50 for ampicillin and penicillin were greater by more than 1 dilution for isolates from CON-CE herds compared with CON-CA and ORG herds, but this relationship did not hold for the MIC90 of these drugs (MIC for CON-CE and ORG herds was greater than that for CON-CA herds). In a univariate analysis, the proportion of penicillin-resistant *S. aureus* isolates was significantly higher in CON-CE herds (76/111; 68.5%) compared to CON-CA (4/99; 4.0%) or ORG herds (32/110; 29.1%). A multilevel model (accounting for clustering of quarter within cow within herd) was made, where the 3 herd types were the main explanatory variable. Other potential variables offered to this model included age of the cow, breed, DIM at time of sampling, SCC at last test, and antimicrobial treatment history for that cow. Results from this multilevel model showed that the proportions of penicillin-resistant *S. aureus* isolates did not differ between the 3 herd types. For analysis of resistance to ceftiofur, sulfadimethoxine, and erythromycin, 3 different groupings of breakpoints were made for each compound. When comparing the proportion of *S. aureus* isolates falling into the 3 different breakpoint groups for ceftiofur resistance, the only significant difference was that there were fewer organic isolates in the middle breakpoint category (1 μg/mL); otherwise, there were no differences in the proportion of isolates falling into the different breakpoint groups from each of the 3 herd types. When comparing the proportion of *S. aureus* isolates falling into 3 different breakpoint groups for sulfadimethoxine resistance, the only significant difference was that there were more organic isolates in the lowest category (32 μg/mL); otherwise, there were no differences in the proportion of isolates falling into the different breakpoint groups from each of the 3 herd types. There were no significant differences between the 3 herd types when comparing the proportion of *S. aureus* isolates falling into 3 different breakpoint groups for erythromycin resistance. For CNS isolates, the MIC50 and MIC90 for ampicillin and penicillin were lower by more than 1 dilution for CNS isolates from organic herds compared to both types of conventional herds; otherwise, these values did not differ by more than 1 dilution between the 3 herd types for the other antimicrobials tested. In a univariate analysis, the proportion of penicillin-resistant CNS isolates was significantly greater in both types of conventional herds (CON-CE, 42/82; 51%; CON-CA, 22/74; 30%) vs. organic herds (14/84; 17%). Similar to the analyses for *S. aureus*, a multilevel model was made to compare penicillin resistance of CNS with herd type as the main explanatory variable. Results from this multilevel model showed that the proportion of penicillin-resistant CNS isolates was significantly greater for CON-CE herds (0.50 ± 0.07) compared to CON-CA (0.31 ± 0.06) or ORG herds (0.17 ± 0.05). When comparing the proportion of CNS isolates falling into 3 different breakpoint groups for ceftiofur resistance, the only significant difference was that there were more organic isolates in the lowest (0.5 μg/mL) and highest (2 μg/mL) categories compared to both conventional herd types; otherwise, there were no differences in the proportion of isolates falling into the various breakpoint groups from each of the 3 herd types. There were no significant differences between the 3 herd types when comparing the proportion of CNS isolates falling into 3 different breakpoint groups for sulfadimethoxine resistance. When comparing the proportion of CNS isolates falling into 3 different breakpoint groups for erythromycin resistance, the only significant difference was that there were more CON-CA isolates in the highest category (≥1 mg/mL); otherwise, there were no differences in the proportion of isolates falling into the different breakpoints from each of the 3 herd types. Importantly, the authors point out that any differences in MIC between isolates from different herd types occurred below clinical breakpoints, so therefore may not affect bacteriological cure rates. Rather unexpectedly, they found bimodal distributions of MIC for ampicillin and penicillin in *S. aureus* isolates from organic herds, suggesting either (1) isolates with a higher MIC are “a natural part of the bacterial population of the bovine mammary gland,” or (2) isolates with higher MIC have persisted within organic herds from a time when antimicrobials were used on the farm.

Dairy farms in the process of transitioning from conventional management to organic certification provide a unique opportunity to study patterns resistance over time after a change in the level of antimicrobial exposure. In addition to comparing conventional and organic farms, Bennedsgaard et al. (2006) followed 19 Danish herds in the process of transitioning to becoming certified organic dairies. These herds were sampled at year 0, 1, and 2 of transition, with quartermilk samples collected from 30 cows at each farm at high risk of infection with *S. aureus* (as determined by a score based on a history of high SCC, breed, and lactation). Herds in the “old organic” category were certified for ≥ 5 years. Antimicrobial exposure for each herd was approximated by calculating the amount of mastitis treatments used in % cows treated/cow-year. The amount of mastitis treatment used by the conventional group was significantly higher than “old organic” herds, but no other significant differences existed between “old organic” herds or the conventional herds in comparison to any of the transition groups (transition year 1, transition year 2, transition year 3) with respect to usage of antimicrobial mastitis treatments. As previously mentioned, the prevalence of penicillin resistance in *S. aureus* and the proportion of penicillin-resistant isolates was similar between “old organic” and conventional herds. Furthermore, no differences were seen in these measures of penicillin resistance between “old organic,” conventional, or any of the 3 transition groups. The same 19 herds were sampled repeatedly over 3 years, and the amount of penicillin resistance among *S. aureus* on these farms did not decrease year after year as they transitioned to organic status. This finding is somewhat unsurprising in light of the fact that antimicrobial usage also was not significantly different. In contrast, Park et al. (2012) found that β-lactam resistance rates of CNS decreased with discontinuation of β-lactam antibiotics in a study following 2 dairies through the process of converting from conventional to organic management over a 3-year period. Composite milk samples were collected from cows at the end of lactation, at freshening, and from cases of clinical mastitis during the last year of conventional dairy production, the transition year, and during the first year of organic production. While still conventional, cows with clinical mastitis were treated with an intramammary product with pirlimycin, and a product with cephapirin, streptomycin and penicillin, or novobiocin and penicillin was given to all cows at dry-off. There was a significant increase in zone diameter for mastitis-associated CNS isolates against cephalothin, cloxacillin, and penicillin when comparing the conventional vs. organic phase. There was no significant change in zone diameter of the other 8 antimicrobials tested. Interestingly, no changes in resistance patterns were seen for mastitis-associated *S. aureus* isolates for the 12 antimicrobials tested. Of importance to note is that the 2 farms in Park et al. were in the US, and therefore antimicrobial usage was completely discontinued at the beginning of the transition to organic status. A similar small-scale case report from Thailand compared AMR of mastitis pathogens before and after the experimental farm’s transition from conventional to organic status for 7 antimicrobial drugs used to treat mastitis (Suriyasathaporn, 2010). All cows in the herd were sampled before beginning the transition, and after 6 months of operating as an organic dairy. The frequency of antimicrobial treatment on the farm decreased from <3 cases/month to > 1 case/month during the study period. Although isolate numbers were small (7 CNS isolates from before transition, 6 from after), a significant decrease was seen in the percent of CNS isolates resistant to gentamycin. Although numeric decreases in percent of resistant CNS isolates were seen for the other 6 antimicrobials, no changes were statistically significant. Data on susceptibility was not reported for *S. aureus* isolates.

1.5 Additional factors explaining variation in antimicrobial susceptibility of staphylococci

Although some evidence exists that conventional vs. organic management may influence the prevalence of AMR in staphylococci causing bovine IMI, this relationship is difficult to tease out from other factors determining the resistance profiles of these mastitis pathogens. This is especially true for NASM (primarily grouped as “CNS” in these studies), where prevalence and type of AMR carriage differs by species. Herd-level management factors, cow-level factors, and geography have all been shown to influence which NASM species may be present or predominant in causing IMI in a particular herd (see below). It is therefore difficult to attribute differences in AMR prevalence of NASM without accounting for this species-level effect. Table 1.2 summarizes work describing the species-specific antimicrobial susceptibility of staphylococci isolates from bovine IMI. The 10 observational studies included describe phenotypic resistance profiles and are limited to work where isolates were identified to species level using genotypic techniques or MALDI-TOF.

When considered as a group, resistance to β-lactam antibiotics is the predominant type of AMR present in staphylococci. The reported proportion of NASM isolates with β-lactamase resistance can be fairly high, with 51.6% phenotypically resistant to penicillin in Argentina (Raspanti et al., 2016), 63% phenotypically resistant to penicillin in South Africa (Phophi et al., 2019), and 80% of CNS isolates positive for the *blaZ* gene (encoding the production of a β-lactamase enzyme) in a study from the Netherlands (Sampimon, 2009). Proportion of phenotypically penicillin-resistant NASM seems to vary geographically, with Nordic countries reporting 34% (Nyman et al., 2018), 23% (Fergestad et al., 2021), and 29% (Persson Waller et al., 2011), while a Korean study found 14% of NASM isolates were resistant to penicillin (Kim et al., 2019) and Nobrega et al. (2018) report a prevalence of 10% in Canada. β-lactam antibiotics are among the few choices for treating mastitis in the US, with first- and third-generation cephalosporins being the most commonly-used mastitis treatments (USDA, 2016; de Campos et al., 2021). Moderate resistance has been observed in NASM against tetracycline, another highly important antimicrobial frequently used in dairy herds, with 30.1%, 20.9%, and 10% of isolates reported to be resistant in Argentina, India, and Canada, respectively (Raspanti et al., 2016; Mahato et al., 2017; Nobrega et al. 2018). This marked geographic variation in resistance patterns may likely be due to differing selective pressure in dairy farm systems around the world. Which specific antimicrobials are most typically used to treat mastitis and in what amount, as well as the various regulation around their usage, varies from country to country.

Studies comparing NASM at the species level have consistently shown that AMR profile varies between species (Sampimon, 2009; Persson Waller et al., 2011; Taponen et al., 2016; Nobrega et al., 2018; Fergestad et al., 2021; Taponen et al., 2023). Overall, both phenotypic resistance and resistance genes are relatively rare in the most common species, *S. chromogenes*, in comparison to other NASM (Sampimon, 2009; Persson Waller et al., 2011). A notable exception is the presence of the *blaZ* gene, which was found in 80% of all 170 CNS isolates and 87% of *S. chromogenes* specifically in a Flemish study (Sampimon, 2009). β-lactamase production was significantly lower for *S. chromogenes* vs. *S. epidermidis* and *S. haemolyticus* in Sweden (Persson Waller et al., 2011). Although a smaller-scale study in Argentina found a relatively high proportion of *S. chromogenes* were resistant to penicillin (45.1%), both *S. haemolyticus* and *S. xylosus* had an even higher proportion of penicillin-resistant isolates (58.6% and 92.9%, respectively; Raspanti et al., 2016). Across a number of studies, authors report that some less-commonly isolated NASM species carried AMR profiles which were the most concerning for public health. Sampimon et al. (2011) found a high prevalence of genotypic resistance (particularly *mecA*) or presence of multiple resistance genes in species with relatively a low prevalence (*S. cohnii, S. equorum, S. fleurettii*, and *S. sciuri*). In Nobrega et al. (2018), resistance to quinupristin/dalfopristin (a combination used to treat serious nosocomial infections in humans) was common in *S. gallinarum* (98% prevalence of resistance among isolates), and *S. cohnii* and *S. arlettae* were frequently resistant to erythromycin (prevalence of 63 and 100%, respectively). The authors specifically highlight *S. arlettae* as worrisome in its AMR profile; it had the highest prevalence of AMR against penicillin (61%), ampicillin (23%), erythromycin (100%), pirlimycin (18%) and clindamycin (99.9%), as well as the highest prevalence of multidrug resistance. A number of studies also call attention to concerning AMR patterns for *S. epidermidis*, which is moderately common in the US and Canada but one of the predominant species found in Nordic countries. In Sampimon et al. (2009), *S. epidermidis* was the second most commonly-found species, it carried multiple resistance genes in ~50% of isolates, and phenotypic penicillin resistance was more common compared to other CNS. The proportion of penicillin-resistant isolates was highest for *S. epidermidis* in a Finnish study compared to other species, with *S. epidermidis* accounting for 6/8 NASM isolates carrying the *mecA* gene (Taponen et al., 2023). Similarly, β-lactamase production was higher for *S. epidermidis* compared to other species (Persson Waller et al., 2011), and it was one of a few species where AMR (including resistance to trimethoprim-sulfonamide) was most frequently observed in Fergestad et al. (2021). Lastly, Taponen et al. (2016) found that *S. epidermidis* was the most resistant among the four major species studied, several isolates were multidrug resistant, and 19% of isolates were *mecA*-positive (encoding for methicillin resistance). Even within a given species, AMR carriage has been linked to certain strain types. For *S. aureus*, carriage of methicillin resistance has been associated with particular clonal complexes both in human medicine (Smith et al., 2021; Garrine et al., 2023) and certain clusters of spa ¬type for bovine clinical mastitis isolates (Freu et al., 2022). The linkage between strain type and AMR is not as well studied for NASM, but Persson Waller et al. (2023b) found that *blaZ* was significantly more common among *S. chromogenes* strains belonging to 2 specific clusters of strain types vs. strains belonging to other clusters.

As AMR carriage differs by species, the particular diversity of NASM responsible for causing IMI on a farm will partly determine the observed herd-level resistance pattern. Various regional and herd-level risk factors have been identified explaining some of the diversity and prevalence of different NASM associated with mastitis and BTM. Different times of year were associated with higher likelihood of IMI for *S. chromogenes*, *S. haemolyticus, S. xylosus,* and *S. warneri* in Dolder et al. (2017), and *S. cohnii, S. simulans, S. sciuri* in BTM in De Visscher et al. (2017). Geographical differences in NASM species diversity among quartermilk samples were found between 4 regions in Canada (Condas et al., 2017a) and 4 states in the US (Jenkins et al., 2019). It is difficult to discern whether these differences are truly a function of geographical variation, or result from farms in a region sharing a similar suite of management practices leading to similar NASM species prevalence and diversity in a herd. Although *S. chromogenes* is the dominant species causing IMI in many countries (as summarized in De Buck et al., 2021), *S. epidermidis* (closely followed by *S. simulans*) was the most commonly-found species in both a Finnish (Taponen et al., 2022) and a Swedish study (Nyman et al., 2018). At the herd level, facility type has been shown to explain some of the diversity of NASM species: cows from herds using a tiestall barn were more likely to have an IMI due to *S. simulans, S. xylosus, S. cohnii, S. saprophyticus, S. capitis,* and *S. arlettae* compared with other NASM species, and less likely to have an IMI due to *S. epidermidis* (Condas et al., 2017a). Cows from herds in Canada using a bedded pack system had a higher relative risk for IMI due to *S. chromogenes* and *S. sciuri* vs. other NASM (Condas et al., 2017a), while Adkins et al. (2022) found *S. cohnii, S. hyicus,* and *S. pseudintermedius* in BTM from sand-bedded freestalls (but not bedded packs), and *S. pasteuri* and *S. piscifermentans* were unique to BTM from bedded packs. In a study by Piessens et al. (2011), sawdust bedding material was associated with IMI due to *S. xylosus* and *S. succinus* for Belgian dairy herds. De Visscher et al. (2017) identified a number of management practices around milking protocol and hygiene associated with the presence of different NASM species in BTM. These include a decreased risk for *S. xylosus, S. simulans*, and *S. chromogenes* in BTM from herds that clip udders, a decreased risk of *S. devriesei* in herds with consistent glove use during milking, an increased likelihood of *S. cohnii* in herds sharing towels between cows when drying udders, and a decreased likelihood of *S. haemolyticus, S. cohnii*, and *S. simulans* in herds that flushed or steamed milking units after use. Hogan et al. (1987) found more IMI due to *S. epidermidis* in herds using no teat dip compared to herds that did, and that *S. hyicus* constituted a greater proportion of staphylococci IMI in herds that used teat dip vs. herds that did not. However, it should be noted that species-level identification of staphylococci in this study was performed using a biochemical test, which may have had limited typeability and accuracy for identification of bovine staphylococci isolates (Vanderhaeghen et al., 2015). Lastly, some herd-level management factors associated with NASM diversity were related to feed and water provided to dairy cows: De Visscher et al. (2017) found an increased likelihood of *S. simulans* in BTM if drinking water for cows was from a public supply (vs. a well), and Petzer et al. (2022) reported proportionally more IMI due to *S. chromogenes* from herds that were pasture-based compared to those that were fed a total mixed ration (TMR), while *S. haemolyticus* was more likely to cause IMI for TMR herds.

Risk factors at the cow level which affect the likelihood of IMI with different NASM have also been identified. Both Thorberg et al. (2009) and Mork et al. (2012) found that *S. chromogenes* was more likely to be isolated from first-lactation animals, while *S. epidermidis* was found more often in third-lactation and older cows. These findings are consistent with 3 other studies reporting *S. chromogenes, S. xylosus*, and *S. simulans* more commonly caused IMI in heifers vs. third-lactation and older cows (De Visscher et al., 2016; Condas et al., 2017a; Nyman et al., 2018). The most likely species to cause IMI also varies within a lactation: Dolder et al. (2017) found that *S. xylosus* was more commonly found in early lactation and *S. warneri* was isolated from mid- to late-lactation animals, while Condas et al. (2017a) report the prevalence of *S. chromogenes*, *S. gallinarum, S. cohnii*, and *S. capitis* to be highest at freshening, and the prevalence of *S. chromogenes* (after an initial decrease from levels at freshening), *S. haemolyticus, S. xylosus*, and *S. cohnii* increased throughout lactation. In Belgian herds, *S. chromogenes* was the predominant species causing IMI both at parturition and throughout lactation; the next most commonly seen species at freshening were *S. sciuri* and *S. cohnii* (De Visscher et al., 2016), while *S. simulans, S. xylosus, S. epidermidis*, and *S. haemolyticus* were the next most common causes for NASM IMI during lactation (Piessens et al., 2011; Supré et al., 2011). Dirty teats have been associated with an increased likelihood of IMI due to *S. cohnii, S. equorum, S. saprophyticus,* and *S. sciuri*, which the authors indicate is consistent with a likely environmental origin for these species (De Visscher et al., 2016). Even physical features of the udder and teats have been associated with different NASM species (De Visscher et al., 2016: quarters with an inverted teat end had higher odds of being infected with *S. chromogenes*, *S. simulans*, or *S. xylosus*; Dolder et al., 2017: udder edema was a risk factor for IMI with *S. chromogenes*).

In addition to unmeasured animal or management-associated risk factors, an important determinate in AMR carriage of mastitis isolates is clonal dissemination within a particular herd. Consistent with behavior of a contagious mastitis pathogen, a certain strain (or strains) of *S. aureus* will predominant for any given herd (Lange et al., 1999; Zadoks et al., 2000; Freu et al., 2022). If the dominant strain of *S. aureus* causing IMI in a dairy herd happens to carry a given AMR determinant, a high proportion of *S. aureus* isolates from that herd will likely exhibit phenotypic resistant against a particular antimicrobial: not solely as a result of environmental pressure and selection, but also as a consequence of phylogeny and the behavior of the pathogen itself. This dominant strain type effect can result in issues of non-independence between isolates from a particular farm (Call et al., 2008), which would be exacerbated in studies enrolling a relatively small number of herds. Pol and Ruegg (2007a) directly address this issue of statistical dependence in their study of 40 herds. In order to avoid dependence between the cow, herd, and exposure category (conventional vs. organic), the authors included only 1 isolate per cow and ≤ 20 isolates per herd in all analyses. Additionally, they report the range of isolates used per herd for each category of mastitis pathogen.

1.6 Why is AMR maintained in organic systems?

In almost all studies summarized in this review, some degree of AMR was found in isolates despite decreased (EU) or absence (US) of selective pressure of antimicrobial use; organic farms in McDougall et al. (2021) had no antimicrobial usage for a range of 7-19 years, with a median of 12 years of organic certification. Assuming there is a fitness cost to bacteria for maintaining AMR genes (Vanacker et al., 2023), this certainly begs the question of why resistance genes have been maintained to any degree in the absence of selective antimicrobial pressures. A rather extreme example of AMR persistence in cattle farms is a study comparing bacteria isolated from retail ground beef raised in conventional and “raised without antibiotics” operations. LeJeune and Christie (2004) identified resistance against chloramphenicol in isolates from both systems, an antimicrobial that had been banned from use in US food animals since 1986. Resistant bacteria remaining on organic farms long after selective pressure of antimicrobial use is gone suggests that other factors play an important role in this long-term persistence. In a study where feedlot steers were fed subtherapeutic levels of antibiotics, Alexander et al. (2008) found that ampicillin-resistant *Escherichia coli* in the control group (no antibiotics) increased due to an evident clonal expansion of an environmental strain (detected by PFGE) during the latter part of this longitudinal study. This environmental strain outcompeted other strains of *E. coli* present in the intestinal tract of the steers in the control group, suggesting that fitness traits beyond carriage of AMR genes play an important role in the prevalence of AMR bacteria. Specifically, the authors suggest that one environmental factor related to the level of AMR was diet, as the prevalence of steers shedding tetracycline-resistant *E. coli* was higher in animals fed grain-based vs. silage-based diets in both treatment and control groups. Although specifically looking at commensal *E. coli* in dairy calves and not mastitis pathogens, one group of researchers set out to explore which factors beyond antimicrobial usage may explain the persistence of an *E. coli* strain (SSuT) in the GI tract which was resistant to streptomycin, sulfonamide and tetracycline (Khachatryan et al., 2004, 2006a, 2006b, 2008; as summarized in Call et al., 2008). Their first study asked if direct antimicrobial selection pressure was maintaining a high prevalence of SSuT *E. coli* strains in calves, and they found that it was not; a clinical trial showed that addition or removal of oxytetracycline from the diet had no effect on the prevalence of SSuT strains in fecal samples over a period of 3 months. Their next step was to ascertain if SSuT traits themselves provide a secondary but unrecognized fitness advantage to these particular strains of *E. coli* by generating null mutants for the SSuT traits (now susceptible to these antibiotics). On average, they found that the null mutant strains retained a competitive advantage over the other susceptible strains, and concluded that the specific genes conferring the SSuT phenotype were not responsible for providing any secondary fitness advantages. At some point between studies, the farm stopped feeding a medicated milk replacer. The researchers observed that after only a short time frame, the SSuT strain had suddenly declined in prevalence. This was unexpected, given that their previous work demonstrated that the SSuT strains had an obvious advantage compared to the susceptible strains. This unexplained decline prompted an additional study, which hypothesized that the milk supplement itself (comprised of dried milk powder, vitamin A and D) was somehow providing an advantage to the SSuT strains. When the milk supplement was reintroduced (both with and without tetracycline), the prevalence of SSuT *E. coli* strains nearly doubled for both groups of animals receiving the milk supplement vs. those that received none. This work highlights an example of a positive selective force (a dietary supplement) in a dairy farm system either directly or indirectly favoring strains of resistant *E. coli*, which was completely unrelated to antimicrobial exposure.

Call et. al (2008) summarize the 3 possible outcomes after exposure to antimicrobials in an individual animal produces a transient increase in AMR prevalence in a population of bacteria, as has been documented to occur in fecal bacteria. Once the negative selective pressure of antimicrobial usage is removed, the first possible outcome is subsidence of AMR in the population, assuming there is a fitness cost to maintaining the AMR traits. Alternatively, if there is no additional fitness cost to maintaining AMR, we would expect to see “eventual displacement in the face of natural turnover of clonal types at the level of individual animals.” A third possibility, as seen in the work from Khachatryan et al., is that there is no (or limited) change in the level of AMR prevalence after selective pressure from antimicrobials is removed. This could occur if AMR traits have been coupled with other some other locally beneficial traits which provide the bacteria possessing them an advantage in their specific environmental niche. Call et al. (2008) illustrate this with a hypothetical model illustrating the effect of antimicrobial exposure in an individual animal (Figure 1.1). First, a transient increase occurs in the relative number of resistant bacteria within a population after exposure to an antimicrobial. During this time of increased replication, there is an increased probability for a genetic event to occur which links AMR carriage to some other trait providing increased fitness in that specific environment. Organisms with the linked AMR carriage and locally advantageous trait survive better in the population, but in the absence of antimicrobial exposure, there is nothing to actively suppress the susceptible strains in the population. Although the relative proportion of bacteria with AMR may decline gradually over time, linkage of AMR to some other advantageous trait could also lead to a gradual increase or maintenance of a baseline prevalence of AMR, even in systems devoid of antimicrobial exposure. So far, work exploring this question has been limited to studying the effect of antimicrobials on resistant bacteria present in the GI tract of cattle. The potential exists for research focused on exploring why maintenance of AMR genes occurs in mastitis pathogens from organic dairies, years after the selective pressure of antimicrobial use has been removed.

1.7 Conclusions

Organic dairy systems provide a novel opportunity in which to identify the antimicrobial resistance patterns of mastitis pathogens experiencing decreased or no selective pressure from antimicrobial use. This narrative review aimed to summarize studies comparing antimicrobial susceptibility of bovine staphylococcal mastitis isolates on organic vs. conventional dairy farms. Numerous factors make direct comparisons of AMR results difficult between studies, including: use of various methods for antimicrobial susceptibility testing and continuously evolving or conflicting schemes for breakpoints; variation in sampling scheme (random vs. targeted sampling of cows, bulk tank milk vs. quartermilk samples, inclusion of isolates associated with clinical vs. subclinical mastitis); differing definitions of “organic” between herds in the EU (where antimicrobial usage is still allowed, but is more tightly regulated and limited) and the US (any animal treated with antimicrobials must leave the herd). Furthermore, studies including a limited number of herds may suffer from a lack of independence between observations. However, the overall conclusions from each study comparing the two different management systems are still informative, as long as the methodology is consistent within a study. Generally, studies comparing the resistance profiles of staphylococci associated with bovine milk samples show that isolates from organic farms are similar or slightly more susceptible to antimicrobials than those associated with mastitis on conventional farms. Although some level of resistance was observed against a number of antimicrobials important for veterinary medicine (cephalosporins, penicillin, tetracycline), overall resistance of mastitis-associated staphylococci is generally low and the most commonly-used mastitis treatments are still effective. A considerable amount of resistance for both NASM and *S. aureus* against penicillin has been described, but the majority of isolates in European and US studies remain susceptible.

Another factor influencing AMR of staphylococci causing mastitis at the herd level is the particular assortment of NASM causing IMI in a herd, as resistance profiles are species-specific. Consequently, different management factors (unrelated to antimicrobial usage) which affect the prevalence and species diversity of NASM on particular farms can indirectly affect the prevalence of observed AMR in a herd. Furthermore, as strain types within species can differ in likelihood of AMR carriage, AMR prevalence may also be a function of predominate strain type(s) in a given herd.

A consistent finding between all studies described was the persistence of resistant mastitis-associated staphylococci on dairy farms which had not used antimicrobials for many years. Some insight on this phenomenon may be gleaned from a theory put forth to explain the observed maintenance of AMR in fecal bacteria in cattle, despite the absence of antimicrobial use. In the transient expansion of a population of resistant isolates following antimicrobial treatment, the likelihood increases that an AMR gene can become linked with some other locally advantageous trait during replication. The selective advantage bestowed on the resistant bacteria could then lead to an increase in their relative abundance and maintenance of the AMR genes over the long-term, provided that the trait linked to AMR continues to afford a selective advantage.

The biggest limitation of most studies comparing resistance profiles of mastitis pathogens between organic and conventional farms is that staphylococci were not identified to the species level. Organisms were primarily grouped as either *S. aureus* or “coagulase-negative staphylococci.” Before MALDI-TOF became more widely available, accurate species-level identification of mastitis-associated staphylococci on a relatively large scale was prohibitively expensive and time-consuming. As resistance profile varies by species, additional work comparing AMR for NASM isolates (while controlling for species) may give further insight into whether resistance profiles differ between management systems for these bacteria. Comparison of predominant strain types within a given species causing IMI between organic and conventional farms could further our understanding of the complex interplay between phylogeny and selection pressures resulting from management factors on AMR of mastitis pathogens. Although researchers were studying fecal *E. coli* and not mastitis pathogens, Walk et al. (2007) found that phylogenetic groupings varied between organic and conventional dairies, suggesting there may be differences between lineages of *E. coli* in their ability or likelihood of acquiring resistance genes. Based on their findings, the authors conclude that “organic farming practices not only change the frequency of resistant strains but also impact the overall population genetic composition of the resident *E. coli* flora.” Additionally, few studies have described resistance patterns of mastitis pathogens before and after transitioning to organic status, and most were limited in both the number of herds enrolled and the amount of time farms were followed. Although likely logistically difficult and expensive, a long-term, larger study of farms transitioning from conventional to organic status would be incredibly valuable in understanding what types of AMR are maintained in organic dairy herds and for how long.

Fortunately, AMR in general remains relatively low in mastitis pathogens from dairy farms. Nevertheless, continued surveillance and further understanding of factors affecting resistance of staphylococci is warranted. Not only are they important pathogens affecting human health, staphylococci are the predominant group of bacteria responsible for mastitis in dairy animals globally. Understanding the complicated interplay of factors affecting AMR in bacterial populations on dairy farms is vital to making science-based decisions around regulations dictating antimicrobial usage. It is in the best interest of the dairy industry to maintain effective antimicrobial treatments that keep cows healthy, decrease animal suffering, minimize production expenses for livestock producers, and allow dairy cows to produce a high-quality product.

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1.9 Tables

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| --- | --- | --- | --- | --- | --- | --- |
| Table 1.1Summary of observational studies comparing antimicrobial susceptibility of staphylococci isolates between organically-managed (ORG) and conventionally-managed (CON) dairy herds. Most studies describe using a combination of morphology, Gram staining, coagulase and catalase test to identify bacterial isolates as *S. aureus* or non-*aureus* staphylococci (NAS)/coagulase-negative staphylococci (CNS). Additional methods for identifying staphylococci to the species level are identified where appropriate. DCT = dry cow treatment; SCC = Somatic cell count; MIC = Minimum inhibitory concentration | | | | | | |
| *Reference; Country*  *Organisms described1* | *Study design and sampling scheme* | *Herd selection considerations*  *Min. no. yr. ORG certified* | *Quantification of AM usage*  *Description of antimicrobials used on farms* | *Susceptibility method2*  *Antimicrobials tested* | *No. isolates tested* | *Selected results* |
| Busato et al., 2000; Switzerland (EU)  *S. aureus*, CNS | Longitudinal (2 herd visits/yr.: 1x on pasture, 1x in confinement); Performed CMT on each lactating cow in herd, quartermilk samples then collected from quarters with CMT >1; Isolates from subclinical mastitis | 152 ORG herds; Stratified random selection (by herd size and farm location by altitude) from herds agreeing to participate; num. herds selected within strata based on actual proportion of herds in each stratum of entire population of Swiss organic dairies  No. yr. ORG herds certified not provided | No quantification of AM usage  65% ORG herds regularly used AM DCT treatment (mostly β-lactam antimicrobials, combinations of β-lactams and other antimicrobials) | *Disk diffusion*  Ampicillin, cefalotin, chloramphenicol, ciprofloxacin, clindamycin, cloxacillin, cotrimoxacol, erythromycin, gentamicin, neomycin, penicillin, rifamycin, tetracycline | *S. aureus*: 37 ORG  CNS: 54 ORG | Data describing the proportion of staphylococci from CON herds resistant to different antimicrobials taken from a previously unpublished survey by the authors (completed 6 years prior).3  Proportions of *S. aureus* isolates from ORG herds resistant to different antimicrobials were similar to those from CON herds (no statistical comparison carried out).  Proportion of CNS isolates from ORG herds resistant to different antimicrobials were similar to those from CON herds, with the exception of a numerically higher proportion of isolates resistant to rifamyin from ORG herds (no statistical comparison carried out). |
| Tikofsky et al., 2003; US  *S. aureus* | Cross-sectional (1 visit/herd); Composite quartermilk samples from each lactating cow in herd; Not specified if isolates from clinical or subclinical mastitis | 22 ORG herds, 16 CON herds; Herds of similar size and geographic distribution selected; All CON herds used blanket DCT  ORG herds certified ≥ 3 yr. ("most much longer") | No quantification of AM usage  On CON herds, β-lactam antimicrobials used most commonly (amoxicillin and pirlimycin most common treatments administered during lactation, penicillin-novobiocin for DCT) | *Disk diffusion*  Ampicillin, cephalothin, erythromycin, novobiocin, oxacillin, penicillin, penicillin-novobiocin, pirlimycin, tetracycline, vancomycin | 261 *S. aureus*: 117 CON, 144 ORG | Strength of association between proportion susceptible/resistant and mgmt. category was evaluated, as well as differences in mean zone diameter for isolates from ORG vs. CON herds.  Differences in antimicrobial susceptibility were observed between *S. aureus* isolates from ORG and CON herds for 7 of 9 antimicrobials studied (results combined over both analyses). *S. aureus* isolatesfrom both types of herds showed good susceptibility to most mastitis antimicrobials, but isolates from ORG herds were significantly more susceptible. |
| Sato et al., 2004; US and Demark (EU)  *S. aureus* | Cross-sectional (1-2 herd visits/yr. for US herds, 1 visit/herd for Danish herds); Bulk tank milk | 30 ORG herds, 30 CON herds from US; 20 ORG herds, 20 CON herds from Denmark; In US, "neighboring" CON herd enrolled as match for each ORG herd; Danish herds chosen randomly  US: ORG herds certified ≥ 3 yr. (mean = 8 yr.); Denmark: ORG herds converted ≥ 9 yr. prior to publication date | No quantification or description of AM usage provided | *Broth microdilution (Sensititre)*  Bacitracin, cephapirin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, oxacillin, penicillin, streptomycin, sulphamethoxazole, quinupristin/dalfopristin, tetracycline, trimethoprim, vancomycin | 483 *S. aureus*: 229 CON, 254 ORG | Overall, antimicrobial susceptibility was very similar between *S. aureus* isolates from ORG and CON herds in both countries. Isolates from CON herds in Wisconsin had significantly reduced susceptibility to ciprofloxacin (vs. isolates from ORG herds), and isolates from ORG herds in Denmark had reduced susceptibility to avilamycin (vs. isolates from CON herds). Differences in antimicrobial susceptibility of *S. aureus* isolates between ORG and CON herds were small relative to differences in isolates observed between the US and Denmark. |
| Bennedsgaard et al., 2006; Denmark (EU)  *S. aureus* | Cross-sectional and longitudinal components; Herds converting to organic farming sampled 3x 1 year apart, CON and ORG herds sampled 1x; Quartermilk samples collected from 30 cows with "high risk of infection" (criteria: history of high SCC, breed, and lactation); Not specified if isolates from clinical or subclinical mastitis | 20 CON herds, 18 ORG herds, and 19 transitioning herds (sampled at 0, 1, 2 yr. of transition); Herds not matched  ORG herds certified ≥ 5 yr. | Estimated mastitis treatments given in % cows treated/cow-year for each of 5 herd grps  CON used more than ORG, but transitioning grps not different from either CON or ORG; Type of AM usage not described | *Blood agar plates with 1 IU penicillin/ml*  Penicillin | 749 *S. aureus* | No statistically significant differences were observed in the prevalence of penicillin resistance in *S. aureus,* or the proportion of *S. aureus* isolates resistant to penicillin between herd groups (ORG, CON, transition year 1, transition year 2, transition year 3). |
| Roesch et al., 2006; Switzerland (EU)  *S. aureus*, NAS | Cross-sectional (1 visit/herd); 5-13 lactating cows (dep. on farm size) randomly selected at 31 DIM (median); Quartermilk samples collected from quarters with CMT ≥ 2+; Isolates from subclinical mastitis | 60 ORG herds, 60 CON herds; ORG herds chosen randomly from interested pool; Matching CON herds selected based on geographic proximity, same agricultural zone (elevation), and farm size  ORG herds certified ≥ 3 yr. | No quantification of AM usage provided, but prophylactic use of AM lower for ORG herds than CON herds  Main AM used for DCT for ORG and CON herds were penicillin (40 and 66%, respectively), cloxacillin (36.5 and 37%, respectively), neomycin (23.5 and 52.7%, respectively), and gentamicin (11.8 and 2.4%, respectively) | *Broth microdilution (custom plates; Sensititre)*  Amoxicillin-clavulanic acid, ceftiofur, chloramphenicol, clindamycin, enrofloxacin, erythromycin, gentamicin, oxacillin, quinupristin-dalfopristin, penicillin, tetracycline, vancomycin | 79 *S. aureus*: 33 CON, 46 ORG  38 NAS: 19 CON, 19 ORG | Percentage of antibiotic resistance did not differ significantly between *S. aureus* and NAS isolates from cows kept on ORG and CON herds for 12 antimicrobials representing either drugs used to treat mastitis in dairy herds, or drugs important in human medicine. The proportion of resistant *S. aureus* isolates was numerically higher from ORG cows (16/46, 35%) vs. CON cows (6/33, 18%), but this difference was not statistically significant. The proportion of resistant CNS isolates was very similar from ORG cows (9/19, 47%) and CON cows (10/19, 53%). NAS isolates had a higher percentage of antibiotic resistance than *S. aureus* isolates. |
| Bombyk et al., 2007; US  Coagulase-positive *Staph.* (CPS), Novobiocin-sensitive CNS (NSCNS), Novobiocin-resistant CNS (NRCNS) | Cross-sectional (1 visit/herd); Composite quartermilk samples collected from "all healthy cows;" Not specified if isolates from clinical or subclinical mastitis | 8 ORG herds, 8 CON herds; All small dairies (20-100 cows), herds not matched  ORG herds certified ≥ 1 year under USDA National Organic Program (no AM usage for ≥ 4 yr.: 1 yr. certified, 3 yr. of transition) | No quantification of AM usage provided  CON herds reported usage of several AM drugs in the past year: cephalosporins (7 herds), penicillins (6 herds), tetracyclines (5 herds) and pirlimycin (5 herds), and 5 herds practiced blanket DCT | *Disk diffusion*  Cefoxitin, cephalothin, erythromycin, novobiocin, penicillin, pirlimycin, tetracycline, vancomycin | 36 *S. aureus*: 9 CON, 27 ORG  210 NSCNS: 55 CON, 155 ORG  159 NRCNS: 102 CON, 57 ORG | Organic dairy management was associated with more overall antimicrobial susceptibility among staphylococci than was conventional management. In an analysis combining all (3) groupings of staphylococci, a larger proportion of isolates from ORG herds were susceptible to pirlimycin and tetracycline compared with those from CON herds. Susceptibility to erythromycin and penicillin did not differ significantly by herd type when all staphylococci were combined (CON vs. ORG).  When broken down by category of CNS (novobiocin susceptible or resistant), isolates within both CNS categories from ORG herds were more likely to be susceptible to pirlimycin than CNS from CON dairies. No difference in tetracycline, erythromycin or penicillin susceptibility was seen between herd types (CON vs. ORG) within either CNS category. A larger proportion of NSCNS vs. NRCNS for both CON and ORG herds were susceptible to tetracycline, leading the authors to suggest that management practices unrelated to antimicrobial use may contribute to the observed differences in susceptibility patterns of CNS on dairy herds. |
| Pol and Ruegg, 2007; US  *S. aureus*, CNS | Cross-sectional (1 visit/herd); Quartermilk samples from a maximum of 50 multiparous cows with no signs of clinical mastitis; Multiparous cows sampled to ensure at least 1 known exposure to intramammary antimicrobial drugs (DCT); Isolates from subclinical mastitis | Herds categorized based on amount of antimicrobial exposure: 20 ORG herds (no usage); 15 conventional–low usage herds (CON-LO) herds not using or using less than or equal to the first quartile of use of each AM compound); 5 conventional–high usage herds (CON-HI) herds using more than the first quartile of a particular AM compound); All herds had 6-mo. avg. bulk tank SCC ≥250,000 cells/mL; CON herds required to have used blanket DCT for at least 5 yr.; Herds not matched  ORG herds certified ≥ 3 yr. | AM usage quantified at both herd and cow level as defined daily dose (DDD).4 Herd-level DDD was calculated by dividing the reported total dose of each drug used per year by the DDD of that AM. Number of DDD was divided by the total number of milking cows to estimate the density of use of particular AM (expressed as number of DDD per lactating cow per year)  β-Lactams, including cephapirin, penicillin, and ceftiofur, were used on the majority of the herds. Cephapirin and penicillin were used as intramammary infusions (treatment of clinical mastitis, DCT). Detailed description of AM usage by drug provided in reference | *Broth microdilution (Mastitis panel; Sensititre)*  Ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin + 2% NaCl, penicillin, penicillin/novobiocin, pirlimycin, sulfadimethoxine, tetracycline | 137 *S. aureus*: 52 CON (15 herds), 85 ORG (18 herds); Range of no. isolates used per herd: CON: 1-9, ORG 1-18  295 CNS: 160 CON (20 herds), 135 ORG (19 herds); Range of no. isolates used per herd: CON: 2-16, ORG 1-16 | Authors took multiple approaches to compare resistance among isolates from the 3 antimicrobial usage groups:   1. Compared proportion for each type of isolate (CNS or *S. aureus*) that was susceptible or resistant in each category (CON vs. ORG) using χ2 test of association, in order to ask if proportion of susceptible isolates independent of herd type 2. Used χ2 test to explore if the MIC for each type of isolate (CNS or *S. aureus*) was independent of herd type (CON vs. ORG) 3. Performed survival analysis of each type of isolates (CNS or *S. aureus*) based on the 3 antimicrobial usage categories (ORG, CON-LO, or CON-HI). Antimicrobial concentrations in wells of the susceptibility test were used as “time,” and event was inhibition of bacterial growth   In order to avoid statistical dependence, only 1 isolate per cow and no more than 20 isolates per herd were included in the analysis. Overall, isolates from ORG herds were more susceptible to antimicrobials than those from CON herds. The authors stress that although some differences were found between antimicrobial groups, most isolates of both types were inhibited at the lowest dilution tested of most antimicrobial drugs.  *S. aureus:*   1. *S. aureus* isolates from CON herds were more likely to be resistant to ampicillin and penicillin compared with isolates from ORG herds. Herd type was not associated with the proportion of resistant isolates for the other antimicrobial drugs tested 2. *S. aureus* isolates from CON herds had a higher MIC for pirlimycin and sulfadimethoxine compared with isolates from ORG herds. Herd type was not associated with the MIC of the other antimicrobial drugs tested 3. In the survival analysis, the MIC that inhibited 90% (MIC90) of *S. aureus* isolates from ORG herds for penicillin and pirlimycin was lower than the MIC90 of the isolates from CON-LO and CON-HI herds (MIC50, the MIC that inhibited 50% of isolates, was not different for these drugs)   *CNS:*   1. CNS isolates from CON herds were more likely to be resistant to ampicillin, penicillin, pirlimycin, and tetracycline compared with isolates from ORG herds. Herd type was not associated with the proportion of resistant isolates for the other antimicrobial drugs tested 2. CNS isolates from CON herds had a higher MIC for ampicillin, pirlimycin, and tetracycline compared with isolates from ORG herds. Herd type was not associated with the MIC of the other antimicrobial drugs tested 3. In the survival curve analysis, the MIC that inhibited 90% (MIC90) of CNS isolates from ORG herds for ampicillin, penicillin, pirlimycin, and tetracycline was lower than the MIC90 of the isolates from CON-LO and CON-HI herds (ORG and CON-LO herds had a lower MIC50 for erythromycin than CON-HI herds, but the MIC90 did not differ by usage group) |
| Garmo et al., 2010; Norway (EU)  *S. aureus*, CNS | Cross-sectional (1 visit/herd); Quartermilk samples from all lactating cows; Isolates from subclinical mastitis | 25 CON herds, 24 ORG herds; All herds Norwegian Red cows; Matching CON herds selected based on herd size (± five cow-years) and type of housing  ORG herds certified ≥ 4 yr. | No quantification of AM usage provided  Generally, Benzyl penicillin and dihydrostreptomycin are the most common antimicrobials used for intramammary treatment in Norway | *Cloverleaf lactamase test*  Penicillin | 132 *S. aureus*: 68 CON, 64 ORG  260 CNS: 167 CON, 93 ORG | Proportions of *S. aureus* and CNS isolates from ORG herds resistant to penicillin were similar to those from CON herds, although no statistical comparison was carried out. Penicillin resistance was proportionately higher in CNS vs. *S. aureus* isolates*.*  *S. aureus:*  6 out of 68 (8.8%) isolates from CON herds were penicillin-resistant, compared with 9 out of 64 (14.0%) from ORG herds.  CNS:  81 out of 167 (48.5%) isolates from CON herds were penicillin-resistant, compared with 93 out of 200 (46.5%) from ORG herds. |
| Cicconi-Hogan et al., 2014; US  *S. aureus*, CNS | Cross-sectional (1 visit/herd); Bulk tank milk | 192 ORG herds, 100 CON herds; Matching CON herds selected based on proximity to ORG herd and herd size category (0–99, 100–199, or ≥200 adult cows)  No. yr. ORG herds certified not provided | No quantification or description of AM usage provided | *Detection of mecA gene by PCR, MRSASelect plates (Bio-Rad Laboratories Inc.)*  β-lactamase resistance (MRSA*Select* plates used to screen for methicillin resistance, and contain a proprietary combination of an unspecified β-lactam, lithium chloride, aztreonam and cycloheximide) | Not provided | 13 isolates from bulk tank milk were identified as methicillin resistant (positive for *mecA* gene): 7 from CON herds, 6 from ORG. Species identification of isolates from bulk tank milk was performed using 16S rRNA and *rpoB* genes.  These 13 isolates were identified as *S. aureus* (n = 1), *S. sciuri* (n = 5), *S. chromogenes* (n = 2), *S. saprophyticus* (n = 3), *S. agnetis* (n = 1), and *Macrococcus caseolyticus* (n = 1). The single methicillin-resistant *S. aureus* isolate was from an ORG herd, for an observed 0.3% prevalence at the herd level. The methicillin-resistant CNS prevalence was 2% in the organic population, and 5% in the conventional population.  The authors highlight the high number of methicillin-resistant *S. sciuri* identified (6 out of 12 methicillin resistant CNS) compared to previous work, and also suggest that a potential methicillin-resistant *Staphylococcus* reservoir in the dairy herd population of the United States may be independent of production system type (CON vs. ORG). |
| Tenhagen et al., 2018; Germany (EU)  *S. aureus* | Cross-sectional (1 visit/herd); Bulk tank milk | 372 CON herds, 303 ORG herds; Minimum herd size 30 lactating cows; Selection of herds based on sampling plan designed to cover German states according to their share of national CON and ORG cow population; Separate sampling plans for the 2 categories as proportion ORG herds comparatively low  No. yr. ORG herds certified not provided | No quantification or description of AM usage provided | *Broth microdilution*  Cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, penicillin, quinupristin/dalfopristin, rifampicin, sulfamethoxazole, streptomycin, tetracycline, tiamulin, trimethoprim, vancomycin | Not provided | Genomic methods used for identifying isolates to the species level (multiplex PCR: 23S rDNA, specific for staphylococci; *nuc* gene, specific for *S. aureus*; *mecA* gene, β-lactam resistance)  Used a binary logistic regression to describe association of methicillin-resistant *S. aureus*-positive samples with herd type (CON vs. ORG), controlling for effect of region and herd size (both significant predictors of MRSA herd status)  The prevalence of MRSA was significantly higher in BTM samples from CON herds (9.7%) compared with ORG herds (1.7%). Proportion of methicillin-resistant *S. aureus* isolates resistant to 12 different antimicrobials tended to be higher from bulk tank milk samples of CON herds (vs. ORG herds). As there were limited number of isolates from ORG herds (n = 5) compared to CON herds (n = 36), no statistical tests were performed |
| McDougall et al., 2020; New Zealand (US organic regulations)  *S. aureus*, CNS | Cross-sectional (1 visit/herd); Quartermilk samples from cows that had had at least 1 lactation, had been treated with DCT (in herds using DCT), had not been treated with any other antimicrobial within 30 d before sample collection, and had an individual SCC of >200,000 cells/mL; Not specified if isolates from clinical or subclinical mastitis | 7 ORG herds, 11 CON herds using ampicillin-cloxacillin DCT (CON-AC), 8 CON herds using cephalonium DCT (CON-CE); CON herds selected on the basis that >50% of cows were treated in each of the 3 previous yr. with 1 DCT product; Herds not matched  ORG herds certified ≥ 3 yr. (median = 12 yr.; range = 7-19 yr.) | Herd-level use of antimicrobials estimated by extracting AM sales data for each herd for the previous 3 yr. to determine total mass of antimicrobials used per kilogram of liveweight per year for each herd, and mass of each class of AM per kg of liveweight per year  β-lactam AM most commonly used DCT products in New Zealand generally, with 25% containing ampicillin, 61% containing cloxacillin, and 13% containing cephalonium, by mass | *Broth microdilution (Mastitis CMV1AMAF; Thermo Scientific)*  Ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, penicillin/novobiocin, pirlimycin, sulfadimethoxine, tetracycline | 320 *S. aureus*: 111 CON-CE, 99 CON-CA, 110 ORG  240 CNS: 82 CON-CE, 74 CON-CA, 84 ORG | Overall, the authors found that the MIC of CNS from ORG herds were lower than isolates from both types of CON herd. However, they point out that these differences in MIC occurred below clinical breakpoints, and therefore may not affect bacteriological cure rates. They found bimodal distributions of MIC for ampicillin and penicillin in *S. aureus* isolates from ORG herds, and suggest either (1) isolates with a higher MIC are “a natural part of the bacterial population of the bovine mammary gland,” or (2) isolates with higher MIC have persisted within ORG herds since antimicrobial usage was occurring on the farm  *S. aureus:*  The MIC50 for ampicillin and penicillin were greater bymorethan1dilutionfor *S. aureus* isolates from CON-CE herds compared with CON-CA and ORG herds, but this relationship did not hold for the MIC90 of these drugs (MIC for CON-CE and ORG herds greater than CON-CA).  In a univariate analysis, the proportion of penicillin-resistant *S. aureus* isolates was significantly higher in CON-CE herds (76/111; 68.5%) compared to CON-CA(4/99;4.0%)orORG herds (32/110; 29.1%). A multilevel model (accounting for clustering of quarter within cow within herd) was made where the 3 herd types were the main explanatory variable. Other potential variables offered to this model included age, breed, DIM, SCC, and antimicrobial treatment history for that cow.  In the multilevelmodel,proportionsofpenicillin-resistantisolatesdidnotdifferbetweenisolates from the 3 herd types.  When comparing proportion of *S. aureus* isolates falling into 3 different breakpoint groups for ceftiofur resistance, the only significant difference was that there were fewer ORG isolatesin the middle category (1 μg/mL); otherwise, there were no differences in the proportion of isolates falling into the different breakpoint groups from each of the 3 herd types.  When comparing proportion of *S. aureus* isolates falling into 3 different breakpoint groups for sulfadimethoxine resistance, the only significant difference was that there were more ORG isolatesin the lowest category (32 μg/mL); otherwise, there were no differences in the proportion of isolates falling into the different breakpoint groups from each of the 3 herd types.  There were no significant differences between the 3 herd types when comparing the proportion of *S. aureus* isolates falling into 3 different breakpoint groups for erythromycin resistance.  CNS:  The MIC50 and MIC90 for ampicillin and penicillin were lower by more than 1 dilution for CNS isolates from ORG herds compared to both types of CON herds; otherwise, these values did not differ by more than 1 dilution between the 3 herd types for the other antimicrobials tested.  In a univariate analysis, proportions of penicillin-resistant CNS isolates were significantly greater in both types of CON herds (CON-CE, 42/82; 51%; CON-CA, 22/74; 30%) than ORG herds (14/84; 17%). Similar to the analyses for *S. aureus,* a multilevel model was also made to compare penicillin resistance with herd type as the main explanatory variable. In this multilevel model, proportion of penicillin-resistant CNS isolates was significantly greater for CON-CE herds (0.50 ± 0.07) compared to CON-CA (0.31 ± 0.06) or ORG herds (0.17 ± 0.05).  When comparing proportion of CNS isolates falling into 3 different breakpoint groups for ceftiofur resistance, the only significant difference was that there were more ORG isolates in the lowest (0.5 μg/mL) and highest (2 μg/mL) categories compared to both CON herd types; otherwise, there were no differences in the proportion of isolates falling into the different breakpoint groups from each of the 3 herd types.  There were no significant differences between the 3 herd types when comparing the proportion of CNS isolates falling into 3 different breakpoint groups for sulfadimethoxine resistance.  When comparing proportion of CNS isolates falling into 3 different breakpoint groups for erythromycin resistance, the only significant difference was that there were more CON-CA isolates in the highest category (≥1 mg/mL); otherwise, there were no differences in the proportion of isolates falling into the different breakpoints from each of the 3 herd types. |
| 1 Terminology used is consistent with authors’ language and groupings of organisms (e.g., NAS vs. CNS) | | | | | | |
| 2 Manufacturer information provided when specified | | | | | | |
| 3 Unpublished survey on antibiotic resistance performed in Swiss dairy farms by the Swiss Federal Dairy Research Station (Schallibaum, 1992) | | | | | | |
| 4DDD is the maximum dose a standard animal (BW = 680 kg) would receive if it were treated following the FDA-approved label dosages | | | | | | |

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| Table 1.2 Observational studies describing species-specific antimicrobial susceptibility of staphylococci isolates from bovine intramammary infections. Ten studies are included which describe phenotypic resistance profiles and isolates were speciated using genotypic techniques or MALDI-TOF. NAS= non-*aureus* staphylococci; CNS = coagulase-negative staphylococci; AMR = antimicrobial resistance; CM = clinical mastitis; SCM = subclinical mastitis | | | |
| *Reference*  *Country* | *Number of isolates1*  *CM or SCM associated* | *Methodology* | *Overall findings* |
| Sampimon et al., 2009  The Netherlands | 170 CNS  Not specified | Broth microdilution; PCR for *blaZ, mecA, ermA, ermB, ermC, msrA, lnuA, msrA, mphC* | Significant differences in resistance patterns were found between CNS species. Phenotypic resistance and resistance genes were relatively rare in *S. chromogenes*, with the exception of *blaZ* (which was present in 80% of all CNS isolates).  For phenotypic resistance, *S. fleuretti* and *S. epidermidis* had the highest resistance to penicillin, oxacillin resistance was most commonly found in *S. fleurettii, S. cohnii, and S. xylosus*, and resistance to macrolide antibiotics was most prevalent in *S. cohnii*, *S. equorum*, and *S. epidermidis.* There was a high prevalence of genotypic resistance (particularly *mecA*) or presence of multiple resistance genes in species with relatively a low prevalence (*S. cohnii, S. equorum, S. fleurettii, S. sciuri*).  The authors note that the resistance profile of *S. epidermidis* was of the most concern; it was the second most commonly found species, carried multiple resistance genes in ~50% of isolates, and phenotypic penicillin resistance was more common compared to other CNS. |
| Persson Waller et al., 2011  Sweden | 154 CNS  Compares clinical and subclinical | Broth microdilution; Cloverleaf β-lactamase test | Overall, prevalence of antimicrobial resistance for CNS was low, but some variation between species was observed. β-Lactamase production was the most common resistance mechanism found, with 29% of isolates found to be positive. The prevalence isolates of producing β-lactamase varied markedly between species. β-lactamase production was significantly higher for *S. epidermidis* and *S. haemolyticus* (40%) compared to *S. simulans* and *S. chromogenes*, where none or only a few of the isolates were β-lactamase positive. Resistance to other antimicrobials besides penicillin was uncommon, and was markedly lower than previous work describing erythromycin, oxacillin and tetracycline resistance levels in CNS. |
| Frey et al., 2013  Switzerland | 408 CNS  Compares clinical and subclinical | Broth microdilution; PCR for *mecA, mecC* | Overall phenotypic resistance: oxacillin resistance (indicator of *mec* gene-mediated methicillin resistance) was the most frequently identified (47.0% of all isolates), and was more frequent in clinical (56.5%) vs. subclinical mastitis isolates (43.9%). In order, the next most common resistances to antimicrobials identified were fusidic acid (33.8% of isolates resistant), tiamulin (31.9%), penicillin (23.3%), tetracycline (15.8%), streptomycin (9.6%), erythromycin (7.0%), sulfonamides (5%), trimethoprim (4.3%), clindamycin (3.4%), kanamycin (2.4%), and gentamicin (2.4%)  Resistance to oxacillin was attributed to *mecA* gene in 9.7% of oxacillin-resistant isolates, while remaining oxacillin-resistant CNS did not contain *mecC* or *mecA1* promoter mutations. Isolates of *S. fleurettii, S. epidermidis, S. haemolyticus,* and *S. xylosus* were identified as carrying the *mecA* gene. Resistance to tetracycline was attributed to the presence of *tetK* and *tetL* genes, penicillin resistance to *blaZ*, streptomycin resistance to *str* and *ant(6)-Ia*, and erythromycin resistance to *ermC, ermB,* and *msr* genes. |
| Taponen et al., 2016  Finland | 400 CNS  Combines clinical and subclinical | Broth microdilution | *S. simulans, S. chromogenes, S. haemolyticus,* and *S. epidermidis* differed in their antimicrobial susceptibility, with penicillin resistance was the most common type of antimicrobial resistance identified. Phenotypic oxacillin resistance was found in all four species (34% of the isolates overall). Whereas the majority of *S. epidermidis* isolates were resistant to benzylpenicillin, only a few *S. simulans* isolates were penicillin-resistant. 21 isolates (5% of isolates overall) were positive for the *mecA* gene (20 *S. epidermidis*, 1 *S. sciuri*).  *S. epidermidis* was the most resistant among the four major species studied, as resistance to antimicrobials was common, several isolates were multidrug resistant, and 19% of isolates were *mecA*-positive (encoding methicillin resistance). |
| Raspanti et al., 2016  Argentina | 219 CNS  Not specified | Broth microdilution | Overall, 51.6% of isolates were resistant to penicillin. The MIC90 value for penicillin was > 8g/ml for CNS isolates included in the study, which the authors note was well above the recommended breakpoint. Fourteen percent of all CNS isolates tested were resistant to oxacillin (of which 16.7% were *mecA* positive), 29.2% to erythromycin and 30.1% to tetracycline. *S. chromogenes* and *S. haemolyticus* showed a very high proportion of isolates resistant to penicillin (45.1% and 58.6%, respectively).The proportion of penicillin-resistant isolates was smaller for *S. warneri* (4/16), and no resistance to oxacillin was observed. In *S. xylosus,* penicillin resistance was the most common among the species tested (13/14 isolates). |
| Mahato et al., 2017  India | 62 CNS  Clinical isolates | Disk diffusion; PCR for *mecA, mecC, vanA* | As a whole, CNS demonstrated a high level of resistance toward oxacillin (85.5% of isolates) and cefoxitin (83.9%), moderate resistance against rifampicin (37.1%), clindamycin (32.3%), erythromycin (25.8%), and tetracycline (20.9%), and a low level of resistance against ciprofloxacin (11.3%) and gentamycin (9.7%). All strains were susceptible to vancomycin, teicoplanin and linezolid. The methicillin resistance gene *mecA* was found in 95.16% of isolates. *S. sciuri* and *S. haemolyticus* had the highest proportion of methicillin resistant isolates. |
| Nobrega et al., 2018  Canada | 1,702 NAS  Combines clinical and subclinical | Broth microdilution (1,702 isolates); whole genome sequencing (405 isolates) | Prevalence of resistance to important antimicrobials highly important frequently used in dairy herds was relatively common (β-lactams: 10%, tetracyclines: 10%), as was resistance to erythromycin (6%), but resistance to antimicrobials critically important for human medicine (vancomycin, fluoroquinolones, linezolid and daptomycin) was rare (<1%). The most frequently identified genetic resistance determinants were mutations in the *folP* gene and MDR efflux pumps; these mutations were present in all NAS isolates and not associated with a multi-drug resistant phenotype. For NAS species intrinsically resistant to novobiocin, specific residues were found in the in *gyrB* gene. The authors were able to link the presence of *blaZ, mecA, fexA*, *erm, mphC, msrA,* and *tet* genes with drug-specific resistance.  In this study, phenotypic antimicrobial resistance patterns were “clearly species-dependent.” Resistance to quinupristin/dalfopristin was common in *S. gallinarum* (98% prevalence), and *S. cohnii* and *S. arlettae* were frequently resistant to erythromycin (prevalence of 63 and 100%, respectively). The authors highlight *S. arlettae* as particularly concerning in its AMR profile; it had the highest prevalence of AMR against penicillin (61%), ampicillin (23%), erythromycin (100%), pirlimycin (18%) and clindamycin (99.9%), as well as the highest prevalence of MDR. Species-specific patterns were also seen in the prevalence of some AMR genetic determinants. *mecA* elements had a 17% prevalence in *S. epidermidis*, but were close to zero for other species. *erm* genes (encoding rRNA adenine N-6- methyltransferases) were found only in *S. epidermidis, S. cohnii, S. equorum*, and *S. chromogenes*. |
| Fergestad et al., 2021  Belgium and Norway | 227 NAS, 45 *S. aureus*  Combines clinical and subclinical | Disk diffusion; PCR for *mecA, mecC* | Staphylococci isolates were analyzed as 3 separate collections from previous studies (1 in Norway, 2 from different regions of Belgium). Over all 3 sample groups, descriptive analyses showed that antimicrobial resistance was more widespread in several NAS species when compared with *S. aureus* isolates (not including MRSA). Resistance to penicillin was most frequently identified in the Norwegian isolate group. Regardless of sample group, AMR was frequently observed in *S. epidermidis* and *S. haemolyticus*. Resistance to trimethoprim-sulfonamide was frequently observed in *S. aureus,* *S. epidermidis*, and *S. haemolyticus*. |
| Taponen et al., 2023  Finland | 244 NAS, 260 *S. aureus*  Not specified | Disk diffusion; PCR for *mecA, mecC, blaZ* | Authors found that penicillin resistance was the only significant form of AMR from staphylococci associated with IMI in Finland, with 18.8% of all isolates (*S. aureus*: 9.3%; NAS: 28.9%) found to be resistant by disk diffusion. Genotypic potential for resistance to β-lactamases was higher, with *blaZ* found in 26.6% of all isolates (*S. aureus*: 18.5%; NAS: 35.2%). In a phenotypic test detecting production of β-lactamases (nitrocefin test), 21.5% of all isolates were positive (*S. aureus:* 11.6%; NAS: 32.0%). Species-specific differences were observed in penicillin resistance, with the proportion of penicillin-resistant being lowest in *S. simulans* and highest in *S. epidermidis*, and *S. epidermidis* accounting for 6/8 NAS isolates carrying the *mecA* gene. |
| Yang et al., 2023  China | 160 CNS, 172 *S. aureus*  Clinical isolates | Disk diffusion; PCR for *blaZ, mecA, mecC, tetK, tetM, ermA, ermB, ermC* | Overall, both phenotypic and genotypic resistance was highest amongst *S. aureus* and CNS for penicillin, followed by erythromycin and tetracycline. Phenotypically, *S. aureus* isolates showed the highest resistance rates to penicillin (58.7%), followed by erythromycin (22.1%), tetracycline (15.1%), gentamicin (10.5%), ciprofloxacin (8.7%), and chloramphenicol (5.8%). CNS isolates displayed high phenotypic resistance to penicillin (71.3%), followed by erythromycin (28.8%), tetracycline (19.4%), gentamicin (9.4%), chloramphenicol (7.9%), ciprofloxacin (2.5%), and cefoxitin (1.3%).  *blaZ* was detected in 61.0% of *S. aureus* isolates, with all penicillin-resistant *S. aureus* isolates positive for the gene. *tetK* and *tetM* were found in 12.2% and 9.9% of *S. aureus* isolates, respectively, with all *tetK/tetM*-positive isolates showing resistance to tetracycline. *ermC* and *ermB* were found in 22.1% and 13.4% of *S. aureus* isolates, respectively, with all erythromycin-resistant isolates carrying *ermC* alone or in combination with *ermB*. No *S. aureus* were positive for *mecA, mecC or ermA.* For CNS isolates evaluated, *blaZ* was found in 69.4% isolates with all showing resistance to penicillin. One each *S. equorum* and *S. saprophyticus* that were resistant against penicillin were negative for *blaZ* but carried *mecA*. *tetK* and *tetM* were found in 17.5% and 12.5% CNS isolates, respectively, with all *tetK*/*tetM*-positive isolates showing resistance to tetracycline. *ermC* and *ermB* were found in 28.1% and 16.9% of CNS isolates, respectively, with all erythromycin-resistant isolates carrying *ermC* alone or in combination with *ermB*. No CNS were positive for *mecC* or *ermA*. |
| 1 Terminology used is consistent with authors’ language and groupings of organisms (e.g., NAS vs. CNS) | | | |

1.10 Figures



Figure 1.1Adapted from Call et. al, 2008. A proposed model illustrating how antimicrobial resistance can be maintained in a farm environment despite the absence of antimicrobial selection pressure, primarily based on studies of resistant bacteria in the GI tract of cattle. Antimicrobial treatment of an individual animal leads to a transient expansion of AMR subpopulations within the gut, as resistant bacteria have a selective advantage. Eventually, the antimicrobial-induced expansion of the resistant population abates when the selective force of antimicrobial use is removed. If there is a fitness cost for maintenance of AMR for an organism, the relative proportion of AMR subpopulations decline in the absence of antimicrobials. However, expansion of the resistant population also increases the likelihood of a genetic event where an AMR gene is linked to another trait, one that confers a niche-specific fitness advantage to the resistant bacteria. If this selective linkage of AMR occurs, maintenance of a baseline prevalence of the AMR subpopulation may occur, despite the lack of selective pressure from antimicrobial use.

CHAPTER 2: Relationship Between Facility Type and Bulk Tank Milk Bacteriology, Udder Health, Udder Hygiene, and Milk Production on Vermont Organic Dairy Farms

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2.1 Abstract

The primary objective of this cross-sectional observational study was to determine whether bulk tank milk quality, udder health, udder hygiene and milk production outcomes were associated with facility type on organic dairies. A secondary objective was to identify other management-related risk factors associated with bulk tank milk quality, udder health, udder hygiene, and milk production on organic dairy herds in Vermont. We aimed to enroll 40 farms, to compare herds using the 2 most common systems (freestalls, tiestalls) for housing organic dairy cattle in the state with those using a bedded pack during the non-grazing season (typically November-May). Two general styles of bedded packs were observed: cultivated bedded packs and untilled deep bedded packs. Due to the limited number of herds using bedded packs to house lactating dairy cattle in Vermont, we combined untilled and cultivated bedded packs to describe udder hygiene, milk quality, and udder health on these loose-housing systems deeply-bedded with organic material. The study was completed on 21 farms (5 bedded packs, 6 freestalls, 10 tiestalls) before interruption due to the COVID-19 pandemic. Data captured from Dairy Herd Improvement Association records from the test closest to the date of the farm visit included average somatic cell score (SCS), standardized 150-day milk (pounds), % cows with current high SCS (SCS ≥4.0), % cows with newly elevated SCS (previous SCS <4.0 to current ≥4.0), and % cows with chronically elevated SCS (SCS ≥4.0 last 2 tests). Multivariable linear regression models were used to describe outcomes by facility type, but suffered from limited statistical power due to small group sample sizes. Unconditional comparisons failed to find statistically significant differences between farms grouped by facility type in metrics captured from Dairy Herd Improvement Association test data, bulk tank milk somatic cell count (BTSCC) and aerobic culture data, or udder hygiene scores. A secondary analysis was conducted using univariate linear regression to identify associations between herd management factors and outcomes for all 21 farms combined. Although not all differences found were statistically significant in this secondary analysis combining all farms, numeric differences that may be biologically important are reported showing farms with deeper bedding had a lower BTSCC, lower newly elevated SCS, lower chronically elevated SCS, lower elevated current SCS, lower average SCS, and better udder hygiene metrics. Farms with lower mean udder hygiene scores had numerically lower chronically elevated SCS, lower elevated current SCS, and lower average SCS. We could not reject the null hypothesis that milk quality and udder health outcomes did not differ by facility type, and this does not preclude the existence of biological differences in these outcomes between facility types. The current study provides insight on factors affecting bulk tank milk quality, udder health and hygiene measures on organic dairy farms in Vermont. Bedded packs may be a viable option for confinement housing during the winter non-grazing season for pasture-based herds interested in a loose-housing system in the Northeastern US, but more research such as longitudinal studies with a larger sample size is needed to test this hypothesis.

2.2 Introduction

Mastitis due to environmental pathogens, such as those commonly found in bedding material, has now become the “most common and costly form of mastitis in modern dairy herds” that have implemented standard mastitis control practices limiting the effect of contagious pathogens (Klaas and Zadoks, 2018). Teats of dairy cattle may be in direct contact with bedding materials for 40 to 60% of the day, making this an important potential source of exposure to opportunistic environmental mastitis pathogens (Tucker and Weary, 2004; Cook et al., 2005; Hogan and Smith, 2012). Work exploring how bedding materials relate to a cow’s risk of contracting mastitis has understandably focused on the most frequently used bedding materials and housing systems in the dairy industry. Currently, the most common type of dairy cattle housing for organic farms in Vermont is a tiestall barn, with freestall barns a distant second (Andrews et al., 2021). As consumer opinion about confinement housing of dairy cattle evolves and influences dairy policy, both the dairy industry and consumers are looking to move away from traditional housing systems that restrict cow movement (Barkema et al., 2015). Many smaller-scale organic dairy farmers in Vermont with aging facilities, and especially tiestall barns, may be looking to adopt a bedded pack system on their farms as a form of loose-housing (Andrews et al., 2021).

The term “bedded pack” encompasses a variety of management styles (Bewley et al., 2017), including compost bedded-packs (CBP), which utilize aerobic decomposition to break down a bedding material of fine wood sawdust or shavings, as well as “conventional,” “traditional,” or “deep bedded packs” (Thurgood, 2009; Benson, 2012; Bewley et al., 2017; The Dairyland Initiative, 2024). CBP can vary in depth, frequency and depth of aeration (tilling), type of bedding material used, and in some regions the inclusion of forced air systems to dry the bedding in situ (Leso et al., 2020). In the Northeastern U.S., some producers are using deep bedded pack systems where large volumes of straw or hay are added daily to an untilled surface and strata of bedding and waste accumulate throughout winter season (Benson, 2012). Oxygen is retained in the system by the selection of bedding material and the timing of its application (Neher et al., 2022; Thurgood et al., 2009). Some authors suggest deep bedded pack barns are synonymous with traditional straw yard housing systems (Bewley et al., 2017; Leso et al., 2020; Ferraz et al., 2020). However, we find the deep bedded packs being constructed for winter housing on organic dairy farms in the Northeastern U.S. differ from traditional straw yards where bedding material is completely removed at approximately monthly intervals and the housing is used year-round (The Dairyland Initiative, 2024; Thurgood et al., 2009; Benson, 2012).

Bedded packs (BP) are perceived to integrate well into Northeastern US pasture-based farm systems, and state and federal agencies in the U.S. are providing financial incentives for dairies to build these structures as part of manure management practices which improve water quality and contribute to soil conservation (USDA-NRCS; Andrews et al., 2021; Thurgood et al., 2009). As interest in BP grows, it is important to better understand milk quality, udder health and hygiene on farms using these housing alternatives. Understanding mastitis risk for cattle housed on BP is especially important for organic dairy farmers, as they have limited effective options for treating intramammary infections (Ruegg, 2009). As mastitis-causing bacteria may thrive in the conditions found in compost bedded-packs (Black et al., 2014), previous work studying mastitis risk and bedding would suggest BP could pose a relatively higher risk for intramammary infections. Loose-housed cows continually add manure to the pack, contributing both pathogenic bacteria (non-*aureus* staphylococci, Wuytak et. al., 2020; *E. coli*, *Klebsiella* spp., and *Enterobacter* spp., Eberhart, 1984; streptococci, Zadoks et al., 2005) and nutrients to the organic bedding material. Organic bedding material is more likely to have a higher bacterial count than inorganic bedding, such as sand, (Hogan et al., 1989; Rowbotham and Ruegg, 2016b), as it supplies nutrients and moisture which encourages bacterial growth. This could lead to higher concentrations of bacteria on teat skin for cows on BP, because: 1) organic bedding (in general) is inherently associated with a higher number of bacteria on teat ends (Fairchild et al., 1982; Rowbotham and Ruegg, 2016b), and 2) a higher concentration of bacteria in bedding is associated with a higher concentration of bacteria on teat ends (Hogan and Smith, 1997; Zdanowicz et al., 2004; Rowbotham and Ruegg, 2016b). This higher concentration of bacteria on teat ends may put the mammary gland at an increased risk of infection, although limited evidence exists for this relationship (Neave et al., 1966; Pankey, 1989; Rowbotham and Ruegg, 2016a).

Previous work describing mastitis risk and cow hygiene on BP systems includes descriptive studies of CBP (Barberg et al., 2007b; Black et al., 2013; Fávero et al., 2015; Eckelkamp et al., 2016b; Albino et al., 2018; Heins et al., 2019). However, research comparing milk quality and cow hygiene between BP and more traditional housing types has so far been limited to freestalls with sand, which is an uncommon housing type for organic farms in Vermont (Andrews et al. 2021). These include a study comparing CBP and sand-bedded freestalls for farms with a history of low bulk tank somatic cell counts (Eckelkamp et al., 2016a), work describing hygiene and bulk tank milk somatic cell count (BTSCC) for sand-bedded freestalls and CBP (Adkins et al., 2022), and a comparison of CBP and 2 types of freestall barns (Lobeck et al., 2011). It is unclear whether the herds included in these prior studies were conventionally-managed or organic dairies. To the best of our knowledge, no studies describe and compare bulk tank milk quality, udder health and hygiene for BP and tiestall barns on small to midsize organic dairies in the same geographic area.

To better inform organic dairy producers in the Northeastern US, who may be interested in using a BP for housing their cattle during the non-grazing season (i.e., for “winter housing,” typically the months of November-May), we conducted a cross-sectional, observational study on organic dairies in Vermont. This study aimed to quantify bulk tank milk bacteriology, udder health and udder hygiene measures for the 2 most common indoor housing systems (freestalls and tiestalls) and farms using a BP among organic farms in Vermont. The objectives of this project were to identify whether bulk tank milk quality, udder health and hygiene outcomes differed by facility type, with a view to determining if BP are a viable option for indoor housing of lactating cows in VT during the non-grazing season. We hypothesized that udder health, hygiene, and bulk tank milk bacteriology of BP herds are inferior to that of more traditional housing types, as has been suggested by some previous research (Barberg et al., 2007b; Lobeck et al., 2011). Therefore, our null hypothesis was that there no association between facility type and udder health, hygiene, and bulk tank milk bacteriology on organic dairy farms using BP and other systems for winter housing of lactating cow in Vermont. A secondary objective was to identify other (non-facility) management-related risk factors associated with bulk tank milk quality, udder health, udder hygiene, and milk production for organic VT dairy herds.

2.3 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).

2.3.1 Herd enrollment and selection

The source population for this study was the farms that responded to a survey sent to all (n = 177) certified organic dairy farms producing cow milk in Vermont in Winter 2018-2019. Certified organic dairy farms in the United States are required to allow their cows daily access to pasture during the grazing season, and cows must obtain 30% of their dry matter intake from grazing (Rinehart and Baier, 2011). In Vermont and other Northeastern US states, forage is unavailable directly from pasture during winter months and the climate necessitates use of indoor housing. When cows have no access to pasture in the winter non-grazing season, organic farms in Vermont house cows in a variety of indoor facility types. Our previous Winter 2018-2019 industry survey quantified the frequency and diversity of indoor housing and bedding types used by organic dairy farmers in the state when cows were not on pasture, and for the current study farms were recruited from the 145 respondents to this survey (Andrews et al., 2021). Dairy farms were eligible for enrollment in the current study if they: 1) responded to the initial survey in the Winter 2018-2019, 2) indicated they met the enrollment criteria of testing with the Dairy Herd Improvement Association (DHIA) at least monthly, 3) milked between 35 and 120 cows, and 4) indicated they would be interested in further participation. Eligible farms were contacted from this source population in Spring 2019 if they responded that they were using 1 of 4 categories of bedding/housing combinations for their indoor housing system: 1) freestall (FS) barn bedded with sand, 2) FS barn bedded with shavings or sawdust, 3) tiestall (TS) barn bedded with shavings or sawdust, or 4) BP. The first 3 housing and bedding combinations are the most frequently used by organic dairies in Vermont to house cows during the non-grazing season, and were compared to BP as they were the housing type of interest for this project. For the purposes of this study, the inclusive term “bedded pack” is used to encompass both CBP and deep bedded packs, and was defined as an enclosed loose housing facility deeply bedded with organic material, in which bedding and waste accumulate throughout the 6–8-month period of time when cows are housed on it and which is only removed once a year. Both CBP and deep bedded packs use carbon-rich substrates to create a clean, comfortable surface which allows animals to move freely. Urine and manure are not removed when bedding material is renewed, in contrast with other housing systems.

A convenience sample of farms was enrolled in Spring 2019 from a list of eligible farms (grouped by housing/bedding combination) using the phone number or email address provided in the 2018-2019 survey response. Our aim was to enroll 40 farms for the current study, with 10 farms from each of the 4 housing/bedding categories described above.

Prior to obtaining the 2018-2019 survey results, based on preliminary data collected by the University of Vermont Center for Sustainable Agriculture Extension group, the study was designed anticipating that it would be possible to enroll 10 organic Vermont dairies using a BP as their primary indoor housing system. However, out of the 17 farms from the 2018-2019 survey that indicated at least some use of a BP, 1 farm was not interested in any further participation, 5 did not use DHIA testing, and 6 only used a BP as a secondary housing system in conjunction with a TS barn, or cows were only on the pack a few hours a day. Because the number of farms using BP was fewer than anticipated, the eligibility requirements were relaxed to include 1 farm where cows spend the majority (two-thirds) of their time in a BP, with the remaining time in a TS with wood shavings. Additionally, 2 BP farms were included that had limited DHIA information: 1 farm did not utilize cow-level testing, and cow-level data for a second farm was limited due to their seasonal lactation schedule. As the number of BP being used in the state to house lactating dairy cattle was less than anticipated, those that were enrolled and grouped together utilized a variety of management strategies. Of the 5 enrolled farms using a BP, 2 would be classified as “compost bedded-packs,” utilizing tilling to promote aerobic decomposition to break down a bedding material of dry, fine wood sawdust or shavings (The Dairyland Initiative, 2024; Bewley et al., 2017; Endres, 2021). These 2 farms bedded solely with shavings/sawdust, adding new bedding only as needed, and cultivated the pack twice a day. Two 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 was added daily to a mass of bedding that accumulates over the 6-8 months cows are housed indoors (The Dairyland Initiative, 2024; Thurgood, 2009; Benson, 2012). The 1 remaining farm fell somewhere between these 2 types of classically defined BP; this farm bedded with straw and woodchips and cultivated every 48 hrs., adding chopped hay and woodchips every time the pack was cultivated. All farms in the study grouped as “bedded packs” shared the qualities of being an enclosed loose housing facility, deeply bedded with organic material (0.9-1.7 meters), which accumulated over the period of time animals were housed indoors and was only removed once a year.

Of the intended 40 herds to be recruited in the study, 21 herds (1 FS bedded with sand, 5 FS bedded with wood shavings/sawdust, 10 TS bedded with wood shavings/sawdust, 5 BP) agreed to participate and farm visits were completed April-May 2019. This study was intended to study cows while they were in their winter (non-grazing months) indoor housing system, so all herds visits were completed before any grazing had begun for the season. Each herd was visited once during the study period. All herds sampled during this period were housing their cows as they would in the non-grazing season. Farm visits were suspended in mid-May 2019 as farms began turning their cows out to pasture, with the intention of resuming in April 2020 to complete the remaining 19 herds. Due to COVID-19 pandemic activity restrictions, the decision was made to not resume the study, and the final analysis included the 21 herds sampled in 2019. As there was only 1 farm sampled using a FS facility bedded with sand, the initial plan to group farms by the 4 housing/bedding combinations specified was abandoned in favor of grouping farms by the 3 facility types used. The single sand FS was combined with FS bedded with wood shavings/sawdust (FS; n = 6), there were 10 TS bedded with wood shavings/sawdust (TS), and 5 BP.

2.3.2 Questionnaire administration, sampling, and udder hygiene scoring

At each farm visit, a questionnaire was administered to collect information about housing and bedding management, as well as other practices on the farm that could impact mastitis risk (Supplemental Material - Questionnaire). The study questionnaire was largely adapted from a previously published survey (Stiglbauer et al., 2013), with additional questions specific to the current study. The questionnaire was reviewed by a social scientist experienced in gathering qualitative data and tested before use with herd managers at the University of Vermont teaching dairy. Questions about mastitis risk explored producer concerns about bedding/mastitis risk; mastitis control, identification and record keeping; milking facilities, procedures, and hygiene practices; information about diet, vitamin and mineral supplementation, and water source; typical calving and periparturient practices; and fly control. Questions about housing and bedding management included describing type of housing system used for both lactating and dry cows; classification and description of any bedding material used; and bedding management practices for each housing type used. The questionnaire also collected some basic herd information (production numbers; number of lactating, dry, and youngstock; breed; record-keeping systems). Farms using BP were asked additional questions to gather detailed information about pack construction, management, monitoring practices, and perceptions comparing BP to any previously used systems. Completion of the questionnaire required 45 minutes on average, ranging from about 30 minutes to 1.5 hours. The questionnaire and interview protocols were registered with the University of Vermont Institutional Review Board (IRB certification 19-0057). The questionnaire was created and administered on a tablet using KoboCollect software (KoboCollect, 2019).

At each farm visit, a bulk tank milk sample was collected directly from the top of the tank using a 250-mL sterile single-use vial (Blue Dippas, Dynalon Products, England) after at least 5 minutes of agitation. Samples were kept on ice in a cooler during transport until they were processed fresh for SCC measurement or were frozen and stored at −20°C in the laboratory, before being sent to a diagnostic lab for microbiological analysis. An on-farm observation sheet was completed, which collected information about the bulk tank, cow identification, a subjective assessment of air quality, and any outdoor exercise area (Supplemental Material – Observation sheet). Additionally, measurements of the housing facilities were recorded for FS and TS where appropriate (stall sizes, pen sizes, bedding depth, stocking density, trainer use), as well as observations about BP when applicable (depth, pen size, and stocking density in m2 per animal). Bedding depth of FS and TS was included as a producer reported value in the questionnaire. Bedding depth of BP facilities was measured by forcing a meter stick down to the level of the cement pad or gravel under the pack, where the pack met a cement knee wall, and recording the height of the pack at that point. Udder hygiene scoring was completed by the same researcher at all farms for a minimum of 30 cows on each farm (the first 30 able to be evaluated in a loose pen, or the first 30 encountered in a TS). A 4-point udder hygiene scoring system was used, where 1 = free of dirt, 2 = slightly dirty (2–10% of surface area), 3 = moderately covered with dirt (10–30% of surface area), and 4 = covered with caked on-dirt (>30% of surface area) (Schreiner and Ruegg, 2002). Animal use for this project was approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC; protocol #PROTO202000089).

2.3.3 Herd-level udder health measurements

Herd-level DHIA test results for the test day closest in time to the farm visit (either preceding or following day of farm visit, whichever was shorter) were captured from the record processing center working with each herd (Lancaster DHIA, Manheim, PA; Dairy One Co-Op. Inc., Ithaca, NY). Information captured included test date, number of lactating cows, standardized 150-day milk production (STD 150-day milk), and test-day average cow-level somatic cell score (SCS). The following udder health measures were also captured from DHIA records: proportion of cows with an SCC ≥200,000 cells/mL on most recent test day (“elevSCS”), where elevated SCS was defined as a somatic cell score of ≥4.0; the proportion of cows with a newly elevated SCS (“newSCS”), which was defined as a SCS changing from <4.0 to ≥4.0 over the last 2 tests; and the proportion of cows with a chronically elevated SCS (“chronSCS”), which was defined as having a SCS ≥4.0 on the last 2 tests (Schukken et al., 2003).

2.3.4 Bulk tank milk culture and bulk tank somatic cell count measures

An aliquot of the bulk tank milk sample was stored at -4°C until it could be transported to the laboratory of a dairy processing plant (St. Alban’s Cooperative/Dairy Farmers of America, St. Albans, VT) within 48 hours of collection for determination of the bulk tank somatic cell count (BTSCC).

Frozen bulk tank milk samples were shipped on ice to the Laboratory for Udder Health (University of Minnesota Veterinary Diagnostic Laboratory, St. Paul) for analysis. Methodology for bulk tank milk cultures at the Laboratory of Udder Health are described elsewhere (Patel et al., 2019). Briefly, thawed, room-temperature bulk tank milk and a 10-fold dilution of each bulk tank milk sample were plated onto MacConkey, Factor (gram-positive selective agar; University of Minnesota), and Focus (selective for streptococci or strep-like organisms; University of Minnesota) media plates and incubated for 2 days at 37°C. Any lactose-fermenting colonies on MacConkey medium were counted and reported as coliform bacteria. Any β-hemolytic colonies on Focus medium were counted and identified to the species level using a MALDI Biotyper (suspect *Streptococcus agalactiae*). All remaining colonies on Focus medium that were not identified as *Strep. agalactiae* were counted and recorded as streptococci or strep-like organisms (SSLO). Hemolytic colonies on Factor medium were counted and identified to the species level using a MALDI Biotyper (suspect *Staph. aureus*). Any hemolytic colonies with a confidence score ≥2.0 for *Staph. aureus* were counted and reported as such. Remaining colonies of staphylococci on Factor media (based on colony morphology, catalase reaction, or Gram stain) were counted and reported as *Staph.* spp. Bulk tank samples were also cultured for *Mycoplasma* spp. (0.1 mL milk was swabbed across a Mycoplasma agar plate, then placed in a 7% CO2 incubator at 37°C for 7 days, after which they were examined for *Mycoplasma* spp. by a trained microbiology technician). For each bulk tank milk sample, total colony-forming units (cfu) per mL were calculated for coliform organisms, *Staph.* spp., SSLO, *Staph. aureus*, *Strep. agalactiae*, and *Mycoplasma* spp. The lower threshold of detection for bacteria in this bulk tank milk culture protocol was 5 cfu/mL, and the upper threshold was 62,500 cfu/mL.

2.3.5 Data management and analysis

Bulk tank milk culture results, BTSCC, DHIA test results, farm-level udder hygiene outcomes, questionnaire data, and farm observations were entered into an Excel database (Microsoft Corp., Redmond, WA). Udder hygiene scores for individual cows were used to calculate 2 farm-level udder hygiene measures: 1) mean udder hygiene score, and 2) proportion of cows with dirty udders (udder hygiene score ≥3), which were incorporated into the database. This Excel database was then imported into the R Statistical Programming Environment (R Development Core Team, 2023) for data cleaning, checking, and statistical analysis. The distribution of outcome variables was assessed to check for normality using a Shapiro-Wilk test with significance set at *P* ≤0.05, visual assessment of distribution and residuals, skewness, and comparison of the median and mean values. Raw bulk tank somatic cell count (BTSCC) data was log10 transformed for analyses. Descriptive statistics were calculated to evaluate the distribution of data, data integrity, and to identify missing data. Descriptive statistics generated included description of general herd characteristics and farm traits, lactating cow housing/facilities, lactating cow bedding material/bedding management practices, milking hygiene procedures, and mastitis control practices for all 21 herds included in the study.

2.3.5.1 Objective 1. Evaluation of relationships between housing system and measures of milk quality, udder health, udder hygiene and milk production.

As most measures of aerobic culture data were not normally distributed even after log transformation, a Kruskal-Wallis test was used to compare cfu counts of bacteria from bulk tank milk between the 3 facility types. Statistical significance for this test was declared at *P* ≤0.05. Multiple attempts were made using multivariable analysis to compare the 4 aerobic culture outcomes for bulk tank milk, but all modeling approaches suffered from over-parametrization even when data were log transformed and were not pursued further.

Independent farm-level predictors from the herd-management questionnaire offered to the multivariable models are described in Table 2.1. Continuous variables underwent correlation analysis to identify predictor variables that were highly correlated (correlation coefficient ≥0.60), and unconditional associations among categorical variables were evaluated using a Pearson’s chi-squared or Fischer’s Exact test as appropriate (*P* ≤0.05). An ANOVA was used to check for correlation between numeric continuous variables and categorical variables (*P* ≤0.05). When a categorical variable had multiple groups with a small number of observations in each, groups were combined when biologically reasonable to have all categories of predictor variables contain at least 5 observations. If any predictor had only 1 observation in a group and there was no way to combine groups in a logical way, it was excluded from further analysis (but listed in descriptive statistic tables, Supplemental Tables S1-S4).

Univariate linear regression was performed in R using the “stats” package to investigate the unconditional relationship between four udder health and production outcomes (BTSCC, avg. SCS, elevSCS, STD 150-day milk) and 2 hygiene outcomes (mean hygiene score, proportion of dirty udders) for each farm and the previously-described herd-level independent variables. The 2 udder hygiene metrics (proportion dirty udders and average udder hygiene score) were used as both predictor variables (in models for other outcome variables) and outcome variables in models of their own. Univariate logistic regression was also performed in R using the “stats” package to investigate the unconditional relationship between chronSCS and newSCS, and the previously-described herd-level independent variables. Any explanatory variable that was unconditionally associated with 1 or more of the 8 outcomes of interest at *P* <0.20 was then offered into a multivariable model (linear for BTSCC, avg. SCS, elevSCS, STD 150-day milk, mean hygiene score, proportion of dirty udders; logistic for newSCS and chronSCS) investigating the relationship between the udder health and production or hygiene outcome and the herd-level predictor variables. If any predictor variables were found to be correlated with each other at the previously described cut-offs, the one with the more highly significant relationship from univariate analysis was offered to the multivariable model when appropriate. The 2 udder hygiene metrics were highly correlated (derived from the same data), so whichever one had a smaller *P-*value from the univariate analysis was chosen for inclusion in the model-building process. Facility type was forced into these multivariable models, as it was the primary explanatory predictor of interest. A backward stepwise variable selection process was then used, with the least significant variables being removed one by one. Final models were selected based on lowest Akaike information criteria, and an *F-*test or likelihood ration test (as appropriate) was used to compare the final model to the model with facility type as the only predictor (significance declared at *P* ≤0.05). Overall statistical significance for facility type (the main predictor of interest) was declared at *P* ≤ 0.05. The multivariable modelling approach described above aimed to investigate the conditional relationship between facility type and the 8 outcomes of interest while controlling for different farm management practices, housing characteristics, milking procedures and mastitis control practices.

2.3.5.2 Objective 2. Identify other (non-facility) management-related risk factors associated with bulk tank milk quality, udder health, and milk production in organic dairy herds.

After grouping all 21 farms together, we used linear regression in the same manner as described above in Objective 1 to explore associations between the independent predictors described in Table 2.1 and 4 udder health and production outcomes (BTSCC, avg. SCS, elevSCS, STD 150-day milk) and 2 hygiene outcomes (mean hygiene score, proportion of dirty udders). Similarly, all 21 farms were grouped together for analysis using logistic regression to explore associations between the independent predictors described in Table 2.1, and the udder health outcomes newSCS and chronSCS. Unconditional relationships between the 8 outcome variables and independent predictors are reported for a significance level of *P* ≤0.20 for an F-test or Z-test (where appropriate), and only for predictor variables with group sizes of at least n = 5.

2.3.6 Power analysis

A priori sample size calculations were not performed, as group size was determined by the number of organic dairy herds housing lactating cows on BP in our region.

2.4 Results

2.4.1 Description of study herds

Of the 21 herds enrolled, 5 used a BP, 1 used a FS bedded with sand, 5 used a FS bedded with shavings/sawdust, and 10 used a TS bedded with shavings/sawdust (Supplemental Table S1). The predominant breeds on all farms were Holstein (n = 8 farms), Jersey (n = 10), and mixed Holstein-Jersey crosses/other (n = 3). The median (mean; range) number of lactating cows was 68 (64.9; 32-99). The median annual rolling herd average milk production for the farms was 6,367 (6,424; 4,082-9,618) kg. Nineteen of the 21 farms tested with DHIA monthly while their cows were in milk, 1 farm tested 5-8 times/year, and 1 tested every other month. On average, DHIA data was captured from a test day 4 days before the farm visit (range: -28 days to +33). The average depth of bedding in the 15 FS and TS where producers provided an estimate was 4.5 cm (SD: 3.5 cm; range: 1.3-12.7 cm). The average depth of bedded packs (measured by researchers) was 130 cm (SD: 31; range: 90-170 cm). Detailed descriptions further characterizing study farm management practices and housing characteristics for lactating animals (e.g., laying surface, ventilation, stocking density), and details about bedding material and bedding management practices for lactating animals (e.g., bedding depth, frequency of adding new bedding, manure removal) are provided in Supplemental Tables S1 and S2, respectively. Detailed descriptions of routine milking procedures and mastitis control practices are provided in Supplemental Tables S3 and S4, respectively.

2.4.2 Description of bulk tank milk quality, udder health measures, milk production, and udder hygiene scores

In a non-parametric, unconditional comparison, there were no statistically significant differences in cfu count between the 3 facility types for any of the 4 bacterial groups measured. However, estimated median values varied numerically between groups (Table 2.2). For example, the median value for streptococci and strep-like organisms among the 10 TS was 167.5 cfu/ml, compared to 32.5 and 35 cfu/ml for FS and BP, respectively. This difference is driven by greater variation in values for TS herds (range: 20 – 1250 cfu/ml). Similarly, the median values of *Staph* spp. cfu counts were numerically higher for TS herds (Table 2.2). None of the 21 bulk tank milk samples were positive for *Strep. agalactiae* or *Mycoplasma* spp. Sixteen of the 21 samples were negative for coliforms on aerobic culture, while 5 farms had a coliform count of 5 cfu/mL. *Staph. aureus* was found in the bulk tank milk from 13/21 herds, with a median (range) cfu/mL of 50 (15-320) when present.

Mean BTSCC, % cows with newly elevated SCS, % cows with chronically elevated SCS, % cows with elevated SCS, avg. SCS, and STD 150-day milk production were numerically similar between the 3 facility types, with overlapping 95% confidence intervals on the mean estimates (Table 2.3).

The overall mean (95% CI) of herd-level udder hygiene scores for all 21 farms was 2.32 (2.16-2.49). The mean hygiene score was 2.2 (1.91-2.44) for BP (n = 5), 2.5 (2.24-2.76) for TS (n = 10), and 2.15 (1.93-2.37) for FS (n = 6). The overall mean proportion of cows with dirty udders in a herd (udder hygiene score ≥3) was 40% (31-48). The mean proportion (95% CI) of cows with dirty udders was numerically higher on TS farms at 49% (35-62), compared to 32% (18-46) for BP farms, and 32% (20-44) for FS farms.

2.4.3 Objective 1. Analysis of relationship between facility type and measures of bulk tank milk quality, udder health, milk production, and udder hygiene scores

Final multivariable models are summarized in Table 2.4. All 21 farms were able to be included in the models for BTSCC, average hygiene score, and proportion of dirty udders. For the models exploring newSCS, chronSCS, and elevSCS, 2 BP farms did not have available DHIA data (n = 19; group sizes: FS =6, TS = 10, BP = 3). One BP farm did not have average cow-level SCS data (n = 20; group sizes: FS = 6, TS = 10, BP = 4). For STD 150-day milk, 1 BP farm and 2 TS farms were missing DHIA data (n = 18; group sizes: FS = 6, TS = 8, BP = 4). Farms with missing data for a particular outcome were excluded for the analyses of that outcome.

2.4.3.1 Bulk tank milk quality outcomes

Variables that were associated at *P* <0.20 with BTSCC in univariate analysis included predominant breed, if herds ever performed culture of mastitic milk, glove use, and herd size. The final multivariable model included facility type (forced) and herd size. Facility type was not associated with BTSCC in the final model (Table 2.4).

2.4.3.2 Udder health outcomes

Herd size category, use of bedding amendment, air quality as assessed by researcher, glove use at milking, and clinical mastitis record keeping practices were offered to a multivariable model for newSCS. The final multivariable model included facility type (forced), air quality and glove use. Facility type was not associated with newSCS in the final model (Table 2.4).

Variables that were associated at *P* <0.20 with chronSCS in univariate analysis included feeding additional supplemental selenium, use of a bedding amendment, clipping/flaming udder hair, clinical mastitis record keeping practices, use of injectable selenium and vitamin E product and proportion of dirty udders. The final multivariable model included feeding a supplemental selenium product, use of bedding amendment, clipping/flaming udders, proportion of dirty udders and facility type (forced). Facility type was not found to be a significant predictor of the outcome chronSCS (Table 2.4).

Bedding amendment use and mean hygiene were offered to a multivariable model for elevSCS. Facility type (forced), bedding amendment, and mean hygiene were retained in the final multivariable model. Facility type was not associated with elevSCS in the final model (Table 2.4).

Feeding additional supplemental selenium, use of bedding amendment, OMRI-listed intramammary product at dry-off, injectable selenium and vitamin E product, and mean hygiene were offered to a multivariable model for herd average SCS. The final multivariable model for avg. SCS included facility type (forced), use of bedding amendment, dry product, injectable selenium, and mean hygiene score. Facility type was not found to be a significant predictor of avg. SCS (Table 2.4).

**2.4.3.3 Milk production outcome**

Variables that were associated at *P* <0.20 with STD 150-day milk included use of injectable selenium and vitamin E product, whether producers cultured high SCC cows, and herd size group. All 3 variables and facility type (forced) remained in the final multivariable model (Table 2.4). Facility type was not associated with STD 150-day milk in the final model (Table 2.4).

**2.4.3.4 Udder hygiene outcomes**

Air quality assessed by researcher was offered to the multivariable model for proportion of dirty udders. The final multivariable model included only facility type (forced), which was not associated with proportion of dirty udders.

Variables that were associated at *P* <0.20 with average hygiene score included whether the producer ever cultured quarter milk samples and whether they checked for cases of clinical mastitis by both examining the udder and forestripping. The final multivariable model included facility type (forced), and how the producer checked for clinical mastitis. Facility type was not associated with the outcome of mean udder hygiene (Table 2.4).

2.4.4 Objective 2. Analysis of farm management factors (non-facility) associated with bulk tank milk quality, udder health, milk production, and udder hygiene scores for all farms combined

Selected results of univariate linear regression models identifying management factors beyond facility type which were unconditionally associated with bulk tank milk quality, udder health, milk production and hygiene outcomes for all farms combined (n = 21) at *P* <0.20 are presented in Table 2.5. We report the results of these univariate regression models as they may be biologically important, even though many failed to reach threshold for declaring statistical significance at *P* ≤0.05, possibly due to our small sample size.

The depth of bedding in stalls for FS and TS herds was unconditionally associated with multiple udder health outcomes. As the depth of bedding in FS and TS herds increased, multiple udder health measures improved, including lower avg. SCS, BTSCC, elevSCS, chronSCS and newSCS. Similarly, comparing farms where cows were on deep bedding (i.e., grouping all herds reporting deeply-bedded stalls plus BP herds) to herds that had stalls with a smaller amount of bedding on top of a mattress or concrete, farms with deep bedding had a numerically lower BTSCC.

Udder hygiene measures were associated with several udder health outcomes. Higher mean hygiene scores and proportion of udders scored ≥3 were associated with higher chronSCS, elevSCS, and average SCS. A few specific management practices were also found to be unconditionally associated with udder health outcomes: consistent glove use was associated with lower newSCS and BTSCC, clipping or flaming udders and parenteral supplementation of vit. E/selenium were associated with fewer chronSCS, and both parenteral supplementation of vit. E/selenium and use of an OMRI-listed intramammary product at dry-off were associated with lower average SCS and higher STD 150-day milk.

Both udder hygiene outcomes were unconditionally associated with the same predictors, most of which were related to the depth of bedding for cows. For the 5 herds using a BP, deeper bedding was associated with lower average hygiene scores and lower proportion of dirty udders. Farms with cows housed on some type of deep bedding (i.e., grouping the 3 FS and TS reporting deeply-bedded stalls, plus the 5 BP herds) had numerically lower average udder hygiene scores and proportion dirty udders compared to cows on stalls with bedding over a mattress or concrete surface. For the fifteen TS and FS reporting bedding depth in stalls, increased bedding depth was associated with lower mean udder hygiene score and a numerically lower proportion of dirty udders.

2.5 Discussion

This work presents the results of our observational study exploring the relationship between facility type and udder health and hygiene metrics, BTM quality (SCC and microbiology), and milk production on organic dairy farms in Vermont. The current study is to the authors’ knowledge the first direct comparison of milk quality, udder health and udder hygiene on BP farms to both TS and FS herds of similar size and management styles, for a population of entirely small to midsize organic dairy farms. The major objective was to identify if milk quality, udder health and hygiene outcomes were associated with facility type, thereby exploring if BP systems are a viable option for housing in Vermont during the non-grazing season compared to the 2 most common indoor housing systems in the state (FS, TS). This study is also the first to describe udder health and hygiene during winter on BP in the Northeastern US, which is significant as the performance of these systems can be greatly influenced by climatic and seasonal factors. We compared BTM bacteriology, udder health and hygiene metrics, and milk yield between BP, TS, and FS herds. There was insufficient evidence to reject our null hypothesis that these metrics do not differ by facility type. However, due to small sample size and limited statistical power, the lack of finding any statistical differences does not rule out the potential existence of biologically important differences between facility types. In fact, for a number of outcomes we found BP herds achieved better results compared to TS herds in the same region. Our findings, while limited due to small sample size, provide observed data to design future studies exploring differences in milk quality outcomes on organic dairy herds using different bedding and housing systems.

2.5.1 Objective 1: Comparison of bulk tank milk quality, udder health, milk production, and udder hygiene measures by facility type

Although there is a substantial body of work describing udder health and milk quality for cows housed in straw yards (Astiz et. al, 2014; Fregonesi and Leaver, 2001; Fregonesi and Leaver, 2002; Ward et. al 2002; Peeler et al. 2000), description of these outcomes in the literature is limited for static deep bedded packs. As such, the focus of the discussion will compare BP in the current study (both static and composting) to the more recent body of work on compost bedded-pack farms. Previous work describing bulk tank milk aerobic culture data for farms using a BP system has primarily been descriptive studies of compost bedded-pack herds (Barberg et al., 2007b; Shane et al., 2010), with one study directly comparing bacterial counts between CBP and FS barns (Lobeck et al., 2012). Although farms in these previous studies used a similar array of bedding materials to those in the current study (wood sawdust, wheat straw by-product, Lobeck et al. 2012; wood sawdust, Barberg et al. 2007; “alternative” organic materials, Shane et al. 2010), the sampling period for these previous works differed from the present in seasonality, compounding the difficulty of direct comparison for milk quality outcomes (Pantoja et al., 2009). Barberg et al., 2007b evaluated milk culture results across the summer months, while Lobeck et al., 2012 sampled year-round; the current study focused solely on sampling during the winter, when organic pasture-based herds are primarily housed inside in Vermont.

The *Staph.* spp. count for the 5 BP farms included in this study was comparable to previous work describing bulk tank milk quality for CBP in Minnesota during the winter months. Lobeck et al. 2012 found a mean of 26.1 cfu/mL (95% CI: 2-443) and Shane et al. (2010) found a range of 0-108 cfu/mL for *Staph.* spp. from BTM in the winter months from 6 CBP farms. Within this highly heterogenous group of bacteria, some species are considered primarily host-adapted (colonizing the skin or udder), while others have been associated with stall surfaces, air, and unused sawdust (Piessens et al., 2011), different facility types (Condas et al., 2017), and environmental contamination and poor teat hygiene at milking time (De Visscher et al., 2016; De Visscher et al., 2017). In general, the use of pre- and post- milking teat dip decreases contamination of bulk tank milk both by commensal skin organisms and environmental contamination at milking time (Hogan et al., 1987, Pankey et al., 1985; Pankey et al., 1987; Quirk et al., 2012). All but 1 farm in the current study would fall into the “low” category for BTM *Staph.* spp. counts (Jayarao et al., 2004), which is consistent with all 21 herds using both pre- and post-dip consistently at milking time.

Streptococci and strep-like organisms counts in BTM for BP in the current study were much lower than those from Minnesota CBP in the winter (98-48,400 cfu/mL, Shane et al. 2010; mean: 911 cfu/mL, 95% CI: 138-6,01, Lobeck et al. 2012). Work from Barberg et al. (2007) describing milk quality on CBP in Minnesota noted that 6 of 12 farms sampled had “high” levels of SSLO. The overall SSLO count for all 21 farms included in the current study was lower than that for the overall *Strep.* count for all facility types studied in Lobeck et al. 2012 (445 cfu/mL, 95% CI: 116-1704). We speculate that milking and bedding hygiene practices among herds included in the current study may best explain this difference in BTM pathogen profiles compared to herds enrolled in prior studies (Jayarao and Wolfgang, 2003), and our results support prior industry guidelines for limiting environmental mastitis pathogen exposure, including monitoring and maintaining udder clealiness (Schreiner and Ruegg, 2003).

All farms had low levels of coliforms in bulk tank milk, indicating excellent hygiene practices at milking time (Jayarao and Wolfgang, 2003). The low BTM coliform counts for BP in the current study are similar to those found for 3 CBP farms in Brazil (2.8 cfu/mL; Fávero et al. 2015). This is in contrast with previous work describing BTM quality for this kind of facility in the U.S. (15-1,128 cfu/mL, Shane et al., 2010; mean: 63.7 cfu/mL, 95% CI: 6-735, Lobeck et al. 2012), although direct comparison of coliform counts between studies may be potentially problematic due to variation in duration of freezer storage (Schukken et al., 1989). Barberg et al. 2007 found that 5 of 12 BP sampled during the summer months had “high” levels of coliforms in BTM, contributing to their conclusion that “special attention to cow preparation procedures at milking time are a must for achieving satisfactory milk quality when cows are housed in compost dairy barns.” Season of the year also effects BTM coliform counts, which are higher in summer seasons (Gillespie et al., 2012). Sampling in late winter may partially explain our findings of lower coliform counts for herds in this study compared to previous studies.

Prevalence of *Staph. aureus* was similar between the 5 VT BP farms in the current study and the 6 described in Lobeck et al. 2012 (6.2 cfu/mL, 95% CI: 1.3-30.1). Farm-level prevalence of *Staph. aureus* was also fairly low for BP studied in Shane et al. 2010 (3 of 6 farms BTM negative) and Barberg et al. 2007 (only 1 of 12 farms with a “high” level of *Staph. aureus*). Overall, the population of all 21 farms in the current study had a higher amount of *Staph. aureus* in BTM than the 18 Minnesota farms described in Shane et al. 2010 (median: 30 cfu/mL, range: 0-320; vs. 17.3 cfu/mL, 95% CI: 3.3-91.2). Although it is not clear how many herds included in previous work on BP were certified organic, this higher prevalence of *Staph. aureus* on organic farms in the current study is consistent with work comparing organic and conventional dairy systems (Pol and Ruegg, 2007).

Analysis of a single bulk tank milk sample from a farm is a simple, convenient, and relatively inexpensive way to capture a snapshot of current milk quality and animal health on a farm, and can be a highly specific (albeit poorly sensitive) screening test for major contagious mastitis pathogens (*Staph. aureus* and *Strep. agalactiae;* Godkin and Leslie 1993). Our bulk tank sampling strategy (collecting a single sample) differed from previous work describing the bacteriology of milk from BP farms, where 4 or 5 consecutive bulk tank milk pickups were collected and then pooled for analysis (Barberg et al., 2007b; Shane et al., 2010; Lobeck et al., 2012). We acknowledge that analysis of a single BTM sample in the current study comes with limitations. Bacterial groups traditionally considered to be primarily environmental in origin (non-*ag. Strep., Staph* spp*.,* coliforms), may enter BTM from cows with an intramammary infection, but also may originate from non-specific contamination (teat and udder skin, bedding, manure, or other environmental sources; Elmoslemany et al., 2009). Furthermore, a single bulk tank sample does not give insight into long-term, consistent patterns of a particular farm’s milk quality as is possible from repeated BTM samplings (Jayarao and Wolfgang, 2003). With the financial constraints of research on commercial dairy farms, the limitations inherent in performing analysis of a single bulk tank milk sample from each farm were a trade-off for the ability to get a picture of milk quality on a larger number of farms included in the study.

The estimates from multivariable models of udder health outcomes included in the current study (percent cows with elevSCS, percent cows with chronSCS, percent cows with newSCS, BTSCC, and avg. SCS) were not statistically different between facility types (Table 2.4). For BTSCC, BP were numerically lower than the other 2 facility types; the difference in BTSCC for BP vs. FS and BP vs. TS equated to an increase of 34,628 and 28,105 cells/mL, respectively, which could amount to an important difference in the bulk tank milk quality premiums under some systems. The odds of a new infection (newSCS) for FS were 10% lower than that for BP; similarly, the odds of a new infection on TS farms were 6% lower than that for BP. Although these estimates represent the relative odds of infection between different facility types and not a proportion of the herd infected, which is often used for industry guidance. Ruegg and Pantoja (2013) propose a benchmark of having <8% of cows developing a new subclinical mastitis infections per month, and Schukken et al. (2003) suggest <10%. Seventy-eight percent of herds, including 3 of the BP herds, enrolled in this study were below those thresholds. The odds of a chronic infection (chronSCS) for FS were 20% higher than that for BP, while the odds of a chronic infection on TS were approximately equivalent to those for BP (1% lower). Although these estimates again represent the relative odds of a chronic infection by facility type, an industry benchmark is to have <10% of cows with chronic subclinical mastitis infections carrying over month to month (U. Minnesota Extension Dairy Team), so the numeric difference seen between BP and FS for this outcome may be biologically important. ElevSCS was numerically lowest for TS herds, while FS herds had a higher proportion of cows with an SCS ≥ 4.0 on current test compared to BP farms. The relative magnitude of the difference for these estimates when compared to BP may be biologically significant (1.8% for FS, -2.4% for TS), as a suggested goal for herds is to have a <15% prevalence for cows with subclinical mastitis (Ruegg and Pantoja, 2013). With regards to numeric difference in avg. SCS, BP farms performed slightly better than FS, and were approximately equivalent to TS. The increase in estimated avg. SCS for FS equates to an increase of roughly 16,250 cells/mL at the cow level, which represents a slight to modest increase in SCC. Although some numeric differences for outcomes were observed in the current study between facility types for newSCS, chronSCS, elevSCS, and avg. LS, given the proportionately large standard errors for all estimates, interpretation of the effect of facility type for these outcomes is challenging.

Some previous work has found BTSCC to be elevated for CBP farms (425,000 cells/mL over all 4 seasons, Black et. al 2013; 325,000 cells/mL during summer, Barberg et. al 2007b). Other groups have found udder health and milk quality measures on CBP farms are similar to farms using more traditional facility types. Specifically, in a study of 18 commercial dairy farms, subclinical mastitis prevalence levels was not statistically different between CBP and 2 types of FS housing in Minnesota and South Dakota, where the percent of cows in a herd with an SCC on test day ≥200,000 cells/mL was 33.4, 26.8, and 26.8% for CBP, cross-ventilated FS, and naturally-vented FS, respectively (Lobeck et al., 2011). Like our study, the sample size of this and other prior studies may have influenced the ability to detect a statistical difference when the observed differences are biologically relevant. Eckelkamp et. al (2016a) found no significant difference in subclinical mastitis prevalence in 8 CBP vs. 7 sand-bedded FS in Kentucky with a history of low BTSCC (21.8 and 19.4%, respectively), as well as no difference in BTSCC between the 2 facility types (229,582 and 205,131 cells/mL, respectively). Subclinical mastitis prevalence was 27.7% for 12 CBP farms in Minnesota (Barberg et. al 2007b), which may be more representative of the general population of BP farms in that state as there were no inclusion criteria around maintaining a low SCC prior to the start of the study. The prevalence of subclinical mastitis for herds in our current study is similar to previous work in the U.S. In contrast, Fávero et. al (2015) found a much higher prevalence of subclinical mastitis (43.8%) and percent new infections (20.9%) for 3 BP farms in Brazil than our study (26 and 7% respectively, for 3 BP with available data).

Cows on BP farms numerically made slightly more milk than those in TS, and were equivalent to those in FS. This increase of 1.7 pounds for BP over TS represents roughly 3% of the average STD 150-day milk production for herds in the study, which is a relatively modest increase in milk production. However, the comparatively large standard errors for both STD 150-day milk estimates make it difficult to interpret the effect of facility type for this metric. Previous research has found no significant differences in various production metrics of cows housed on BP vs. in FS barns (Lobeck et al., 2011; Eckelkamp et al., 2016a; Costa et al., 2018). Varying production metrics for cows housed on BP have been reported previously (kg/cow/day, fat-corrected milk/cow/day, average L/cow/day, ME-305, rolling herd average, energy-corrected milk), preventing direct comparisons of milk production between the BP in the current study and other work. Additionally, many variables play a role in determining milk production (nutrition, breed, seasonality, DIM), so teasing out the effect of facility type alone on production in an observational study is difficult. However, as Leso et. al (2020) point out, “results in the literature indicate that high levels of milk production are possible in CBP.” As BP potentially improve cow comfort, one might expect improved milk production compared to traditional confinement housing systems (Calamari et al., 2009; Ruud et al., 2010).

TS farms had numerically higher proportion of dirty udders and avg. udder hygiene score, while FS and BP systems were similar. However, interpretation of these numerical differences is difficult, given that the standard errors for all 4 estimates are large relative to the coefficient estimates. Previous work found that cow hygiene on BP systems was comparable to traditional facility types in the Upper Midwestern U.S., Southeastern U.S., and Brazil (Barberg et al., 2007b; Shane et al., 2010; Black et al., 2013; Eckelkamp et al., 2016b; a; Costa et al., 2018; Adkins et al., 2022; Andrade et al., 2022). Black (2013) and Eckelkamp (2016a) reported that increased pack moisture allows wet bedding material and manure to adhere more easily to animals, meaning that cow hygiene is dependent on conditions of the BP. This sentiment was echoed by the BP producers in the current study, who shared that keeping their cows clean during periods of wet or humid weather could be a challenge. However, all BP in the current study had an average udder hygiene score of less than 2.5, and the farm with the lowest mean average udder hygiene score overall was a BP. Although Cook (2002) identified challenges of comparing dairy cattle hygiene between different facility types, we chose to focus on gathering observations of udder hygiene. The relationship between udder hygiene and health is well-studied, and was a tractable observation to make during non-grazing season farm visits where individual animals were often roaming freely in a pen, or confined in a TS barn.

2.5.2 Objective 2: Analysis of farm management factors (non-facility) associated with bulk tank milk quality, udder health, milk production, and udder hygiene scores for all farms combined

One finding from the univariate analysis combining all 21 farms is that farms with deeper bedding had more favorable udder hygiene metrics. When comparing farms that housed cows with a deep bedding system (deeply-bedded stalls or a BP) to those that housed cows on stalls with a smaller amount of bedding (over a mattress or concrete surface), the deeply-bedded systems tended to have better hygiene scores. This agrees with previous observational field studies of FS barns, including: Cook et al. 2016 (prevalence of dirty udders 13% lower for farms using deep bedding vs. stalls with mats), de Vries et al. 2015 (deep-bedding vs. mat/mattress reduced the likelihood of a cow having a dirty hindquarter by half), and Robles et al. 2020 (farms with mattress-based stalls had a higher prevalence of cows with dirty upper legs/flanks vs. those using a deep bedding system, often inorganic sand). In contrast, an experimental study looking at the effect of bedding depth in TS over 28-day periods found no difference between leg, flank, and udder hygiene of cows using deeply-bedded stalls (14 cm) and the control treatment (2-3 cm; Wolfe et al., 2018).

Beyond comparing udder hygiene of cows housed on a deep-bedding system to cows that were not, there was a linear association between bedding depth (depth of BP, depth of bedding in FS and TS) and hygiene score. As the measured height of bedding got deeper (height of BP, or amount of bedding material in stall), cows tended to have cleaner udders. To the best of our knowledge, work exploring this direct relationship between measured bedding depth and hygiene is limited to a single study by de Vries et al. 2015, who found no relationship between prevalence of dirty hindquarters and 3 different FS bedding height groups (<0.56 cm, 0.56–1.75 cm, >1.75 cm). This relationship between bedding depth and udder hygiene was especially strong for BP in particular, although our sample size was limited at 5 herds. To the best of our knowledge, this specific association has not previously been explored for BP herds. There is opportunity for future research looking at this relationship between increased amount of bedding used in deep-bedded systems (or more deeply-bedded stalls) and the benefit of improved udder hygiene and milk quality.

Multiple measures of udder health in this study were associated with udder hygiene, in accordance with the well-supported tenet that better cow hygiene is associated with better milk quality. The association between hygiene and udder health has been well-documented, both at the cow level (for IMI presence: de Pinho et al. 2012; for SCS/SCC: Reneau et al. 2005, Dohmen et al. 2010, and Sant’anna et al. 2011; for both SCS and IMI: Schreiner and Ruegg, 2003) and at the herd-level (BTSCC: Barkema et al. 1998; new IMI rate: Cook et al. 2002; average herd SCC, incidence clinical mastitis, and % new high SCC: Dohmen et al. 2010). Of particular relevance to the current work, a study carried out on 3 BP farms in Brazil found the odds of a new case of subclinical mastitis (SCC ≥200,000 cells/mL) and of a cow having subclinical mastitis on test day increased 32% and 16%, respectively, for each 1-unit increase in leg cleanliness score, (Fávero et al., 2015). Although leg cleanliness score was associated with both mastitis outcomes on 3 Brazilian BP, udder hygiene score was not, which should not be interpreted to suggest a better biological significance of either of these alternative hygiene scoring systems.

We also found from the univariate regression results farms using deeper bedding had better milk quality outcomes. Although there is an established recommendation of 15 cm for deep bedding of FS (Bickert, 2000; Cook, 2002) and limited studies exploring ideal bedding material depth for TS barns (Tucker and Weary, 2004; Tucker et al., 2009), these prior studies focused on cow comfort outcomes. As stated in a literature review by McPherson (2020), "very little research has investigated the effect of bedding depth on cow cleanliness” or considerations around udder health outcomes. We speculate that the relationship between deeper bedding and better udder health outcomes seen in our current work is mediated through the presumed causal pathway of (1) deeper bedding leading to improved hygiene, and (2) improved hygiene resulting in better udder health. Although recommending a particular depth may prove difficult as there are many contributing factors which are particular to a producer’s barn and bedding source, the opportunity still exists for research exploring optimal stall bedding depths of different organic materials with a focus on mastitis and udder health outcomes.

As for any observational study, there is the potential for bias to have influenced the observed results. Most importantly, participating herds were not a random sample of organic farms in the state, possibly resulting in selection bias. Participating herds were a convenience sample of a subset who responded to our initial survey in Winter 2018-2019 (source population). The potential exists that producers who volunteered to participate in the current study are systematically different in some way with regards to their management practices compared to the general population of organic farms in Vermont. 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, 2022). Herds in the current study were slightly smaller, averaging 65 cows per farm, but with higher-producing cows (7,828 kg milk/cow/year, estimated from captured DHIA records). For comparison, the average dairy cow in the U.S. produced an average of 10,926 kg of milk in 2022 (Progressive Dairy, 2017). It is important to acknowledge that organic cows on average produce less milk (Stiglbauer et al., 2013), and with decreased milk production comes decreased susceptibility to mastitis (Grohn, 2000). This relationship may in part explain the relatively low prevalence of mastitis occurring on these farms in comparison to the general population of dairy farms. Lastly, cross-sectional studies are unable to demonstrate causality for associations presented between management practices and outcomes. However, these limitations are inherent to every observational study, and all attempts were made to control for potential confounding with the multivariable models presented.

Perhaps the biggest limitation of the current study is the small number of farms in each facility type, which limited statistical power. As state agencies had been promoting the use of BP systems for years in Vermont, we had anticipated it would be feasible to enroll 10 farms using this system to house their lactating animals. This turned out not to be the case; the Winter 2018-2019 survey showed that many dairy farms were instead using these systems for non-lactating animals (heifers, dry cows; Andrews et al. 2021). Furthermore, the COVID-19 pandemic precluded resumption of the study in Spring 2020, limiting the number of farms included to herds sampled in 2019, and not all farms had DHIA data for every outcome of interest. A related limitation is that well-established mastitis control practices were widely adapted by participating herds, so we were unable to analyze associations between certain practices and BTM quality, udder health, and hygiene. A large body of work exists showing consistent udder health benefits from using these and other practices, so lack of association between these fundamental mastitis control practices and desirable outcomes in the current study should not be taken as evidence that they provide no benefit. As group sizes for each facility type were limited, we would caution against making inferences from the findings beyond the source population of this study. The potential still exists for future studies with a larger number of farms enrolled to further characterize milk quality and udder health on BP systems in the Northeastern US. By enrolling farms from a larger geographic area, future studies may be able to enroll a larger number of BP farms, increasing the statistical power needed to identify particular management factors which are beneficial or detrimental on BP specifically. Our data may be used to inform new hypotheses and power calculations for future study design.

While BP systems are not common for housing lactating cows in Vermont, farms using this system in the state are using both compost bedded-packs managed with daily cultivation and untilled deep bedded pack systems. As untilled and cultivated bedded pack systems differ in numerous regards (Leso et al., 2020), the initial goal was to enroll enough farms using each type and treat them as separate groups in the analysis. As the relatively small number of BP used in our state to house lactating dairy cattle created a challenge for enrolling 10 herds using this kind of system in our observational study, it was necessary to combine both types of system in order to achieve our objective of describing udder hygiene, milk quality, and udder health on these loose-housing systems deeply-bedded with organic material. While we acknowledge that grouping them together is not ideal, this diversity is a reflection of how the target population (small-medium, pasture-based organic dairy farms) are actually using them in the Northeastern U.S. (Benson, 2012). Despite this limitation, including bedded pack farms managed in a variety of ways sheds light on a broader spectrum of options used within this loose-housing system. Our current study demonstrates that farms can achieve excellent milk quality using either an untilled, deep bedded pack system or a tilled aerobically composting bedded pack system for indoor housing; 3 of the 5 BP farms had a BTSCC ≤99,000 cells/mL, and the remaining 2 were ≤160,000 cells/mL. Furthermore, the lowest BTSCC in the study (54,000 cells/mL) was a static BP farm using woodchips and straw. This low BTSCC was not achieved by selectively dumping milk from high-SCC cows; this farm also had the lowest overall % cows with elevated SCS (8.6%; data not shown).

BP systems have a number of advantages for producers considering updating their facilities, including a smaller initial investment when compared to a new FS or TS barn (Barberg et al., 2007a; Janni et al., 2007; Black et al., 2013), although the cost year-over-year for bedding is substantial (Shane et al., 2010). Bedded packs are designed for cow comfort (Barberg et al., 2007b; Bewley et al., 2012), and prevalence of lameness, foot, and leg injuries in these systems has been found to be less than TS and FS barns (Barberg et al., 2007b; Lobeck et al., 2011; Burgstaller et al., 2016). Lastly, manure management and environmental stewardship is a top concern for both dairy producers and the general public (Holly et al., 2018). Anecdotally, the BP producers enrolled in the study were pleased with their systems of manure management, viewing their used bedding material and manure as a valuable soil amendment and an integral part of their nutrient management plan. Bedded pack systems decrease the amount of liquid manure waste when compared to conventional barns, and the used bedding with manure is more easily composted before use as a soil amendment. As aged pack material is drier before it is spread on fields, it poses less of a risk for run-off into waterways, increases soil infiltration of nutrients, and creates flexibility around timing of manure application to fields (Rushmann, 2023). Bedded packs may be a good housing option for small, pasture-based farms in the Northeastern U.S. when properly managed on farms with excellent milking hygiene practices already in place. However, more research is needed to explore how udder health, milk quality, udder hygiene and milk production compares to more traditional housing systems.

2.6 Conclusion

In an observational study of 21 organic dairy herds in Vermont we found no statistical differences in milk quality and udder health outcomes between herds using different bedding and housing management systems. For 5 of the 6 studied udder health and production metrics, and both udder hygiene measures, BP either performed slightly better numerically or were approximately equivalent in comparison to the most commonly-used facility types for organic dairy cows in Vermont. However, the relatively large standard errors for most of these estimates and the lack of finding a statistical difference does not rule out possible biologically important effects of facility type for these outcomes. Our results may be due to the small group size for each facility type. Bedded packs may therefore be a viable option for pasture-based herds looking for a loose-housing system, but future studies enrolling larger number of farms using each type of housing are needed to more definitively explore these relationships. Findings from the secondary analysis of results supported the established tenets that better cow hygiene is associated with better milk quality, and farms with deeper bedding had more favorable udder hygiene metrics. Additionally, farms using deeper bedding had better milk quality outcomes, which may likely be mediated through improved hygiene resulting in better udder health outcomes.

2.7 Acknowledgements

This project was funded by the USDA NIFA OREI project 2018-51300-28561. We would like to thank all the organic dairy producers who agreed to participate in this study, for giving us their time and allowing us to collect samples from their farms. We would also like to thank Jennifer Timmerman and the Laboratory for Udder Health at Veterinary Diagnostic Lab (University of Minnesota), as well as the laboratory staff at St. Alban’s Cooperative/Dairy Farmers of America, for their advice and analyses of bulk tank milk samples collected in the study. The authors have not stated any conflicts of interest.

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2.9 Tables

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| --- | --- | --- | --- |
| Table 2.1Predictors offered to multivariable models for each of the 8 different outcomes of interest along with facility type (forced) | | | |
| Predictor | | | Level of parameter, if categorical: |
| Farm demographics/lactating cow housing | | |  |
|  | Facility type | | Bedded pack; Freestall; Tiestall |
|  | Predominant breed | | Holstein; Jersey/Other |
|  | Herd size (lactating cows) | |  |
|  | Herd size group (lactating cows) | | 30-55; 56-69; 70-100 |
|  | Subjective assessment of air quality (producer) | | Excellent; Good; Fair/Poor |
|  | Subjective assessment of air quality (researcher) | | Good; Fair |
|  | Age of facility (years) | |  |
|  | Feed supplemental vit. E and selenium | | Yes; No |
| Lactating bedding management practices | | |  |
|  | Lying surface for cows1 (deeply-bedded vs. not) | | Deeply-bedded stalls or bedded pack; Stalls with bedding on a mattress or concrete surface |
|  | *If use shavings/sawdust/*  *woodchips for bedding material:* | |  |
|  |  | Moisture-content | Kiln-dried; Fresh/raw |
|  | Bedding amendment (e.g., hydrated lime) used on surface | | Yes; No |
|  | *If facility is freestall or tiestall:* | |  |
|  |  | Freq. adding new bedding to stalls (times per week) |  |
|  |  | Freq. scraping stalls (times per week) |  |
|  |  | Depth bedding in stalls (cm) |  |
| Mastitis control and milking hygiene practices | | |  |
|  | Clip/flame udder hair | | Yes; No |
|  | Keep record of clinical mastitis events | | Always; Sometimes/Temp.; Never |
|  | Routinely culture mastitic milk | | Always/Sometimes; Never |
|  | Routinely culture high somatic cell count cows | | Always/Sometimes; Never |
|  | Ever perform culture of mastitic cows | | Yes; Never culture |
|  | Use intramammary product at dry-off (OMRI-listed) | | Yes; No |
|  | Parenteral supplementation with vit. E and selenium | | All lactating cows regularly/ Occasionally as needed; No |
|  | Glove use at milking | | All milkers consistently; Inconsistently/No |
|  | Check for clinical mastitis by noticing abnormal cow/abnormal udder and forestripping | | Yes; No |
|  | Type of milking system used3 | | Parlor; Tiestall |
| Farm-level udder hygiene metrics | | |  |
|  | Average udder hygiene score | |  |
|  | Prop. dirty udders (%; udder hygiene score ≥3) | |  |
| 1 If freestall or tiestall, producer asked if used deeply-bedded stalls | | | |
| 2 OMRI: Organic Materials Review Institute | | | |
| 3 One freestall farm used an automated milking system | | | |

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| --- | --- | --- | --- | --- | --- |
| Table 2.2 Objective 1: Descriptive and univariable results for bulk tank milk aerobic culture outcomes by facility type [median (range)]. *P-*value is for Kruskal-Wallis test by facility type grouping | | | | | |
| Bacteria group (cfu/mL) | Overall (n = 21) | Bedded packs (n = 5) | Tiestalls (n = 10) | Freestalls (n = 6) | *P-*value |
| *Staph.* spp. | 65 (0-665) | 40 (0-130) | 85 (15-665) | 67.5 (5-125) | 0.62 |
| *Strep.* and strep-like orgs. | 45 (10-1250) | 35 (10-80) | 167.5 (20-1250) | 32.5 (25-260) | 0.10 |
| *Staph. aureus* | 30 (0-320) | 0 (0-30) | 47.5 (0-320) | 42.5 (0-100) | 0.19 |
| Coliforms | 0 (0-5) | 0 (0-5) | 0 (0-5) | 0 (0-5) | 0.82 |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Table 2.3 Objective 1: Descriptive results for milk quality, udder health and production outcomes by facility type [mean (95%CI)] | | | | |
| Outcome | Overall | Bedded packs | Tiestalls | Freestalls |
| BTSCC (log10cells/mL) | n = 21 | n = 5 | n = 10 | n = 6 |
|  | 5.13 (5.06-5.20) | 5.00 (4.84-5.17) | 5.14 (5.05-5.23) | 5.21 (5.09-5.33) |
| % newly elevated SCS1 | n = 19 | n = 3 | n = 10 | n = 6 |
|  | 5.7 (4.2-7.3) | 7.0 (2.8-11.2) | 5.4 (3.0-7.8) | 5.6 (3.0-8.3) |
| % chronically elevated SCS1 | n = 19 | n = 3 | n = 10 | n = 6 |
|  | 13.6 (11.2-16.1) | 14.5 (5.4-23.7) | 14.3 (11.9-16.7) | 12.0 (6.7-17.3) |
| % SCS ≥ 4.0 current test1 | n = 19 | n = 3 | n = 10 | n = 6 |
|  | 24.9 (21.6-28.3) | 26.0 (12.6-39.3) | 25.4 (22.1-28.6) | 23.7 (16.9-30.5) |
| Avg. SCS2 | n = 20 | n = 4 | n = 10 | n = 6 |
|  | 2.44 (2.26-2.62) | 2.38 (1.84-2.91) | 2.45 (2.31-2.59) | 2.50 (2.00-2.93) |
| Standardized 150-day milk (pounds)3 | n = 18 | n = 4 | n = 8 | n = 6 |
|  | 50.0 (45.7-54.3) | 46.9 (39.8-53.9) | 49.4 (43.1-55.7) | 53.0 (43.5-62.5) |
| 1 DHIA data not available for 2 bedded pack farms | | | | |
| 2 DHIA data not available for 1 bedded pack farm | | | | |
| 3 DHIA data not available for 1 bedded pack farms and 2 tiestall farms | | | | |

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| --- | --- | --- | --- | --- |
| Table 2.4 Objective 1: Final multivariable models describing the relationship between facility type (forced) and milk quality, udder health, production, and udder hygiene outcomes | | | | |
| Outcome | Explanatory variable | Group (sample size) | Coefficient estimate (SE) | *P-*value |
| BTSCC (log10cells/mL) | |  |  |  |
|  | Intercept |  | 4.8 (0.15) |  |
|  | Facility type (forced) | Freestall (n = 6) | 0.19 (0.09) | 0.05 |
|  |  | Tiestall (n = 10) | 0.16 (0.08) | 0.07 |
|  |  | Bedded pack (n = 5) | Ref. | Ref. |
|  | Herd size | All herds (n = 21) | 0.003 (0.002) | 0.15 |
| % newly elevated SCS | |  |  |  |
|  | Intercept |  | -3.8 (0.55) |  |
|  | Facility type (forced) | Freestall (n = 6) | -0.11 (0.40) | 0.79 |
|  |  | Tiestall (n = 10) | -0.07 (0.38) | 0.86 |
|  |  | Bedded pack (n = 3) | Ref. | Ref. |
|  | Subjective assessment air quality (researcher) | Good (n = 14) | 0.99 (0.43) | 0.02 |
|  |  | Fair (n = 5) | Ref. | Ref. |
|  | Glove use at milking1 | Never/Inconsistently (n = 9) | 0.63 (0.30) | 0.03 |
|  |  | Always (n = 9) | Ref. | Ref. |
| % chronically elevated SCS | |  |  |  |
|  | Intercept |  | -2.8 (0.37) |  |
|  | Facility type (forced) | Freestall (n = 6) | 0.18 (0.43) | 0.68 |
|  |  | Tiestall (n = 10) | -0.01 (0.32) | 0.97 |
|  |  | Bedded pack (n = 3) | Ref. | Ref. |
|  | Feed supplemental vit. E and selenium2 | Yes (n = 11) | 0.20 (0.29) | 0.50 |
|  |  | No (n = 7) | Ref. | Ref. |
|  | Use bedding amendment | Yes (n = 5) | 0.55 (0.32) | 0.08 |
|  |  | No (n = 14) | Ref. | Ref. |
|  | Clip/flame udder hair | Yes (n = 5) | -0.55 (0.31) | 0.07 |
|  |  | No (n = 14) | Ref. | Ref. |
|  | % udder hygiene scores ≥3 | Herds with available data (n = 19) | 1.8 (0.61) | 0.003 |
| % SCS ≥ 4.0 current test | |  |  |  |
|  | Intercept |  | 0.85 (10.6) |  |
|  | Facility type (forced) | Freestall (n = 6) | 1.8 (5.7) | 0.75 |
|  |  | Tiestall (n = 10) | -2.4 (5.3) | 0.66 |
|  |  | Bedded pack (n = 3) | Ref. | Ref. |
|  | Use bedding amendment | Yes (n = 5) | 8.0 (4.2) | 0.07 |
|  |  | No (n = 14) | Ref. | Ref. |
|  | Mean hygiene | Herds with available data (n = 19) | 9.8 (4.7) | 0.06 |
| Avg. SCS | |  |  | 20 |
|  | Intercept |  | 0.93 (0.44) |  |
|  | Facility type (forced) | Freestall (n = 6) | 0.38 (0.21) | 0.09 |
|  |  | Tiestall (n = 10) | 0.03 (0.19) | 0.86 |
|  |  | Bedded pack (n = 4) | Ref. | Ref. |
|  | Use intramammary product at dry-off (OMRI-listed) | Yes (n = 5) | -0.30 (0.16) | 0.08 |
|  |  | No (n = 15) | Ref. | Ref. |
|  | Use bedding amendment | Yes (n = 5) | 0.52 (0.16) | 0.007 |
|  |  | No (n = 15) | Ref. | Ref. |
|  | Parenteral supplementation vit. E/selenium | Regularly or occasionally (n = 9) | -0.36 (0.14) | 0.02 |
|  |  | No supplementation (n = 11) | Ref. | Ref. |
|  | Mean hygiene | Herds with available data (n = 20) | 0.64 (0.19) | 0.005 |
| Standardized 150-day milk (pounds) | |  |  | 18 |
|  | Intercept |  | 41.2 (6.1) |  |
|  | Facility type (forced) | Freestall (n = 6) | -0.06 (7.0) | 0.99 |
|  |  | Tiestall (n = 8) | -1.7 (6.6) | 0.80 |
|  |  | Bedded pack (n = 4) | Ref. | Ref. |
|  | Parenteral supplementation vit. E/selenium | Regularly or occasionally (n = 7) | 7.0 (5.2) | 0.20 |
|  |  | No supplementation (n = 11) | Ref. | Ref. |
|  | Culture high SCC cows | Always/Sometimes (n = 8) | 9.3 (5.9) | 0.14 |
|  |  | Never (n = 10) | Ref. | Ref. |
|  | Herd size grp. (lact. cows) | 70-100 (n = 8) | -0.18 (7.3) | 0.98 |
|  |  | 56-69 (n = 5) | 10.3 (6.2) | 0.12 |
|  |  | 30-55 (n = 5) | Ref. | Ref. |
| % udder hygiene scores ≥3 | |  |  |  |
|  | Intercept |  | 0.32 (0.08) |  |
|  | Facility type (forced) | Freestall (n = 6) | 0.002 (0.11) | 0.99 |
|  |  | Tiestall (n = 10) | 0.17 (0.10) | 0.12 |
|  |  | Bedded pack (n = 5) | Ref. | Ref. |
| Avg. udder hygiene score | |  |  |  |
|  | Intercept |  | 2.3 (0.17) |  |
|  | Facility type (forced) | Freestall (n = 6) | -0.04 (0.21) | 0.84 |
|  |  | Tiestall (n = 10) | 0.33 (0.19) | 0.11 |
|  |  | Bedded pack (n = 5) | Ref. | Ref. |
|  | Check for clinical mastitis by noticing abnormal cow/abnormal udder and forestripping | Yes (n = 8) | -0.25 (0.16) | 0.14 |
|  |  | No (n = 13) | Ref. | Ref. |
| 1 One farm used automatic milking system | | | | |
| 2 One farm unable to provide response | | | | |

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| --- | --- | --- | --- | --- | --- |
| Table 2.5 Objective 2: Selected models of univariate analysis identifying (non-facility type) factors unconditionally associated with milk quality, udder health, production, and udder hygiene outcomes at *P* <0.20 | | | | | |
| Outcome | Explanatory Variable | Group (sample size) | Coefficient estimate (SE) | *P-*value | Intercept |
| BTSCC (log10cells/mL) | |  |  |  |  |
| Model 1 | Lying surface | Mattress or concrete (n = 13) | 0.12 (0.07) | 0.12 | 5.1 |
|  |  | Deep bedding (n = 8) | Ref. | Ref. |  |
| Model 2 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -0.02 (0.01) | 0.11 | 5.2 |
| Model 3 | Glove use at milking2 | Never/Inconsistently (n = 9) | 0.10 (0.07) | 0.19 | 5.1 |
|  |  | Always (n = 11) | Ref. | Ref. |  |
| % newly elevated SCS3 | |  |  |  |  |
| Model 4 | Glove use at milking | Never/Inconsistently (n = 9) | 0.58 (0.29) | 0.05 | -3.1 |
|  |  | Always (n = 9) | Ref. | Ref. |  |
| Model 5 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -0.13 (0.07) | 0.06 | -2.4 |
| % chronically elevated SCS3 | |  |  |  |  |
| Model 6 | Clip/flame udder hair | Yes (n = 5) | -0.37 (0.25) | 0.13 | -1.8 |
|  |  | No (n = 14) | Ref. | Ref. |  |
| Model 7 | Parenteral supplementation vit. E and selenium | Regularly or occasionally (n = 8) | -0.31 (0.19) | 0.11 | -1.7 |
|  |  | No supplementation (n = 11) | Ref. | Ref. |  |
| Model 8 | % udder hygiene scores ≥3 | Herds with available data (n = 19) | 1.26 (0.48) | 0.01 | -2.4 |
| Model 9 | Avg. udder hygiene score | Herds with available data (n = 19) | 0.63 (0.25) | 0.01 | -3.3 |
| Model 10 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -0.05 (0.04) | 0.17 | -1.7 |
| % SCS ≥ 4.0 current test3 | |  |  |  |  |
| Model 11 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -1.2 (0.42) | 0.01 | 30 |
| Model 12 | % udder hygiene scores ≥3 | Herds with available data (n = 19) | 13.6 (8.5) | 0.13 | 19.6 |
| Model 13 | Avg. udder hygiene score | Herds with available data (n = 19) | 7.7 (4.3) | 0.09 | 7.1 |
| Average SCS4 | |  |  |  |  |
| Model 14 | Parenteral supplementation vit. E and selenium | Regularly or occasionally (n = 9) | -0.27 (0.18) | 0.15 | 2.6 |
|  |  | No supplementation (n = 11) | Ref. | Ref. |  |
| Model 15 | Use intramammary product at dry-off (OMRI-listed) | Yes (n = 5) | -0.29 (0.21) | 0.18 | 2.5 |
|  |  | No (n = 15) | Ref. | Ref. |  |
| Model 16 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -0.05 (0.03) | 0.10 | 2.6 |
| Model 17 | % udder hygiene scores ≥3 | Herds with available data (n = 20) | 0.75 (0.45) | 0.12 | 2.1 |
| Model 18 | Avg. udder hygiene score | Herds with available data (n = 20) | 0.39 (0.23) | 0.11 | 1.5 |
| Standardized 150-day milk (pounds)5 | |  |  |  |  |
| Model 19 | Parenteral supplementation vit. E and selenium | Regularly or occasionally (n = 7) | 9.0 (4.5) | 0.06 | 46.5 |
|  |  | No supplementation (n = 11) | Ref. | Ref. |  |
| Model 20 | Herd size | Herds with available data (n = 18) | 0.26 (0.14) | 0.07 | 33.1 |
| % udder hygiene scores ≥3 | |  |  |  |  |
| Model 21 | Depth of bedded pack (m) | Bedded pack herds (n = 5) | -0.5 (0.06) | 0.004 | 0.97 |
| Model 22 | Lying surface | Mattress or concrete (n = 13) | 0.17 (0.08) | 0.06 | 0.30 |
|  |  | Deep bedding (n = 8) | Ref. | Ref. |  |
| Model 23 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -0.02 (0.02) | 0.13 | 0.54 |
| Avg. udder hygiene score | |  |  |  |  |
| Model 24 | Depth of bedded pack (m) | Bedded pack herds (n = 5) | -0.96 (0.15) | 0.008 | 3.4 |
| Model 25 | Lying surface | Mattress or concrete (n = 13) | 0.33 (0.16) | 0.06 | 2.1 |
|  |  | Deep bedding (n = 8) | Ref. | Ref. |  |
| Model 26 | Depth of bedding in stalls (cm)1 | Tiestalls and freestalls (n = 15) | -0.06 (0.03) | 0.07 | 2.6 |
| 1 Stall bedding depth for freestalls and tiestalls bedded with wood shavings or sawdust | | | | | |
| 2 One farm used automatic milking system | | | | | |
| 3 DHIA data available for n = 19 herds. | | | | | |
| 4 DHIA data available for n = 20 herds. | | | | | |
| 5 DHIA data available for n = 18 herds. | | | | | |

CHAPTER 3: Staphylococci and mammaliicocci: which species are important for udder health on organic dairy farms?

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3.1 Abstract

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

3.2 Introduction

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

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 also differ in how they behave as intramammary pathogens; the ability to cause persistent infections varies by species (Nyman et al., 2018; Valckenier et al., 2021), as well as the presence of antimicrobial resistance determinants (Frey et al., 2013; Fergestad et al., 2021), virulence potential (Naushad et al., 2019; França et al., 2021), and interaction with a host’s immune system (Åvall-Jääskeläinen et al., 2013; Breyne et al., 2015).

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

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

3.3 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).

3.3.1 Sample origination

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

3.3.2 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).

3.3.3 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.

3.3.4 Speciation of bacterial isolates

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

3.3.4 Determination of IMI status and selection of data set

Using the bacteriological status and speciation information, a quarter-day IMI status was assigned to each quarter observation: 1) “no growth,” 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 no growth *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 3.1 depicts the selection of the final data set of quarter-day observations using these criteria.

3.3.5 Statistical analysis

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

A linear hierarchical repeated measures mixed model was fitted to the data set in order to compare SCS of quarters infected with a single SaM species to no growth 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 no growth 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).

3.4 Results

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

The initial data set included 3,331 quarter-level observations, with 22 different species of staphylococci and mammaliicocci identified. SaM species causing IMI excluded from further analyses due to having < 5 IMI observations included: *M. fleurettii, M. sciuri, M. vitulinus, S. auricularis, S. capitis, S. cohnii, S. epidermidis, S. gallinarum, S. hominis, S. pseudintermedius, S. saprophyticus,* and *S. succinus*. The final data set consisted of 2,260 observations: 648 quarters with an IMI due to 10 different SaM (each causing at least 5 IMI), and 1,972 no growth quarters. Observations included in the final data set came from 1,272 quarters of 360 cows across all 10 herds included in the field study. The mean (median; range) number of cows included per herd was 36 (36; 34-39), whereas the number of quarters included per cow was 3.5 (2; 1-4). The mean number of observations per quarter included was 2.1 (2; 1-4). Twenty-seven percent of observations were the sole observation contributed to the data set by a given quarter, 41% came from quarters contributing 2 time points, and 31% and 1% came from quarters contributing 3 and 4 observations, respectively. The average time elapsed between sequential observations of a quarter was 37.1 days (median: 34.5; SD: 11.6), with an overall range of 27-96 days.

*S. chromogenes* was the most frequent species (59% of quarter observations with a SaMIMI), followed by *S. aureus* (17%)*, S. haemolyticus* (6%)*,* and *S. simulans* (5%)*.* A large amount of variability was observed in the SCS for no growth 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 3.2). The observed SCS for *S. chromogenes* IMI ranged from -2.6 to 8.9 (median: 3.3; equivalent to 2,000 cells/mL to 6.1 million cells/mL), with 29.7% of observations having a SCS ≥ 4.0. The observed SCS for *S. aureus* IMI ranged from 0.6 to 10.5 (median: 5.9; equivalent to 8,000 cells/mL to 18 million cells/mL), with 87.5% of observations of having an SCS ≥ 4.0. The observed SCS for *S. haemolyticus* IMI ranged from -2.1 to 6.1 (median: 3.5; equivalent to 3,000 cells/mL to 880,000 cells/mL), with 33.3% of observations having a SCS ≥ 4.0. The observed SCS for *S. simulans* IMI ranged from -0.8 to 6.7 (median: 3.4; equivalent to 7,000 cells/mL to 1.3 million cells/mL), with 37.1% of observations having a SCS ≥ 4.0.

In a model comparing SCS of quarters infected with SaM to no growth quarters and adjusted for DIM with an interaction term between IMI status and parity, the interaction between IMI status and parity was not significant (*P* = 0.86); thus, effect of the quarter IMI status on SCS was the same, regardless of parity for this data set. In a model comparing SCS of quarters infected with SaM to no growth 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 no growth quarters with DIM as a fixed predictor (as a third degree polynomial variable) are presented in Table 3.1. Somatic cell score was significantly higher in quarters infected with *S. agnetis, S. aureus, S. chromogenes, S. devriesei, S. haemolyticus, S, hyicus, S. simulans, S. warneri, and S. xylosus* compared to uninfected quarters (Table 3.1).

Least square means estimates of quarter SCS across DIM for the ten different SaM modeled as compared to no growth quarters are presented in Figure 3.3. 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 no growth quarters (Figure 3.3).

Predicted raw SCC for quarters infected with different SaMspecies at 91 days in milk are presented in Table 3.2. The highest cell count was for quarters infected with *S. warneri,* followed by *S. aureus, S. agnetis,* and *S. hyicus* (Table 3.2)*.* 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 3.2).

3.5 Discussion

The current study describes how quarter-milk SCS varied as a result of IMI with the most frequently isolated SaM from a longitudinal, cross-sectional study of 10 certified organic dairy farms in Vermont, US. The relative distribution of various SaM and their effect on qSCC was similar to previous studies reporting data for conventionally-managed dairies. *S. chromogenes* was the most frequent species, followed by *S. aureus, S. haemolyticus,* and *S. simulans*. A large amount of variability was observed in qSCC for no growth 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 no growth quarters. The highest cell count was for quarters infected with *S. warneri*, followed by *S. aureus, S. agnetis*, and *S. hyicus*.

*S. chromogenes* was the most frequently identified SaM associated with subclinical IMI on 10 organic dairy herds in Vermont. This is consistent with other studies using genotypic methods or MALDI-TOF for speciation of SaM isolates from both conventional (De Visscher et al., 2016; Condas et al., 2017a; Rowe et al., 2019; Wuytack et al., 2020) and organic (Peña-Mosca et al., 2023) herds in various countries. In contrast to other research focused on SaM epidemiology and similar to Peña-Mosca et al. (2023), we included *S. aureus* IMI data in our analysis. This was motivated by two factors: 1) *S. aureus* has previously been identified as a pathogen of particular concern on organic dairy farms in the US (Ruegg, 2009), and 2) *S. aureus* IMI would serve as a relevant reference category for effect of IMI on SCS (in addition to no growth/negative control quarters). In agreement with Peña-Mosca et al. (2023), the second most frequently isolated SaMspecies among these ten herds was *S. aureus.* Distribution of the next most frequently found species (in order, *S. haemolyticus, S. simulans, S. agnetis,* *S. warneri*, *S.* *devriesei*) in the current study was most similar to previous work on SaM in the US and Canada (Condas et al., 2017a; Rowe et al., 2019). Interestingly, *S. equorum*, *S. cohnii,* *S. hominis,* and *M. sciuri* were all commonly-found SaM species in Belgian studies (De Visscher et al., 2016; Wuytack et al., 2020), but were infrequently found in the current study and not included in the final data set. As the farms in the current study were all certified organic dairies, the ecology of intramammary pathogens (including the diversity of SaMspecies found) could potentially differ from that of conventional farms. We suggest this is possible because, in addition to extent of antibiotic use, differences in management factors exist between conventional and organic dairies (Stiglbauer et al., 2013), and various management factors appear to affect the diversity of SaM species found (Dufour et al., 2012; Condas et al., 2017a). However, we found that the relative distribution of various SaM species in this population of small to midsize organic farms was similar to previous studies conducted on conventionally managed dairies.

In agreement with previous research describing the effect of SaM species on qSCC (using isolates from multiple herds and genotypic methods or MALDI-TOF for species identification), most of the frequently found species from this population of organic dairy farms increased qSCC above that of no growth quarters. Fry et al. (2014) also found *S. chromogenes, S. simulans, S. xylosus, S. haemolyticus, S. warneri,* and *S.* *hyicus* had a higher qSCC than no growth quarters, as well as *S. capitis* and *S. epidermidis,* two species which were not isolated in great enough numbers from milk samples in the current study to be included in the analysis. Isolates used in Fry et al. were a subset of a larger population from quarter-milk samples collected by the Canadian Bovine Mastitis and Milk Quality Research Network, described by Condas et al. (2017b). This larger study also found the same six SaM species previously listed increased quarter SCC above that of no growth quarters, as well as the other species identified in the current study (*S. aureus, S. agnetis*). It may be important to note that at the time of publication of Fry et al., *S. agnetis* had not yet been described as a distinct staphylococcal species; isolates of this species were likely present in milk samples included in that study, but not identified as such*.* While Condas et al. (2017b) found *S. equorum* to elevate quarter SCC above that of no growth quarters, we did not in this current study. The low number of *S. equorum* IMI observations in our study may have limited our ability to observe an effect on qSCC. Of the 17 SaM species included in Condas (2017b), *S. equorum* had the second lowest quarter SCC (40,800 cells/mL); the only species with a lower qSCC was *S. hominis*, which did not differ from no growth 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 no growth quarters; with the exception of *S. arlettae,* these species were isolated from IMI in the current study but were not present in high enough numbers to be included in the analysis. Although the scope of species included in Supré et al. (2011) was more limited, they also found that IMI due to *S. aureus, S. chromogenes, S. xylosus,* and *S. simulans* resulted in a higher SCC compared to noninfected quarters. One species not previously compared to no growth quarters in these aforementioned studies is *S. devriesei,* which we found significantly elevated quarter SCC above that of no growth quarters. As the SaM on these organic farms are under different selective pressures than those causing IMI on conventional farms, there is the potential that a given species may differ in its effect on qSCC and interaction with the host. For example, if dominant *S. chromogenes* strains differed between conventional and organic herds, the potential effect on qSCC could differ as well. Our current study does not test this hypothesis. Although the effects on quarter SCC for SaM on these organic dairies is similar to those previously described on conventional farms, comparisons between the studies should be made with caution, and the potential exists to design future studies comparing virulence factors and antibiotic resistance determinants of SaM isolates causing IMI on conventional vs. organic dairy farms.

The predicted SCC for quarters infected with *S. aureus* stayed above 200,000 cells/mL across the entire range of observed DIM (Figure 3.3), 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 3.2), which was determined from 15 quarter observations. This extends the findings of Fry et al., where the geometric mean SCC for quarters with *S. warneri* was 233,200 cells/mL (95% CI: 90,400-601,600), from 9 quarter observations. The small number of isolates for this species likely resulted in the large 95% confidence intervals of predicted SCC for *S. warneri* seen in both studies. For two studies including larger number of observations for *S. warneri,* quarter SCC estimates stayed well below the 200,000 cells/mL cut-off (for 31 observations in Condas et al., 2017: 63,270 cells/mL, 95% CI: 42,010-95,280; for 105 observations in Taponen et al., 2022: 52,000 cells/mL, 95% CI: 38,000–71,000). In the current study, the predicted qSCC for *S. chromogenes, S. agnetis, S. hyicus, S. simulans,* and *S. xylosus* only became elevated over 200,000 cells/mL late in lactation (286, 208, 261, 270, and 281 DIM, respectively). This effect of DIM is not unexpected, given that SCC normally increases even in no growth quarters towards the tail-end of lactation (Schepers et al., 1997). While still elevated significantly above that of no growth quarters, those infected with *S. devriesei* and *S. haemolyticus* stayed below this threshold throughout the range of DIM assessed for each species. In the observed data, SCS for quarters with an IMI due to *S. chromogenes* and *S. aureus* had significant overlap; this was similar to work by Woudstra et. al (2022), who reported quarter-level SCC by SaM on one dairy in Sweden. Additionally, Supré et al. (2011) found that *S. chromogenes*, *S. simulans*, and *S. xylosus* induced an increase quarter SCC comparable with that of *Staphylococcus aureus* for 3 farms in the Netherlands, while controlling for DIM, parity, milk production, and herd. More recent research from the same group found that the SCC from quarters with a persistent IMI due to *S. chromogenes* was comparable to SCC of quarters infected with a major pathogen such as *S. aureus* (Valckenier et al., 2021)*.* However, in our current study, this overlap in effect on SCC was no longer apparent for the least square means estimates of quarters infected with *S. aureus* and *S. chromogenes*, and accounting for the effects of DIM and repeated observations.

Within a given SaM species, there was considerable variability in the observed quarter SCC (Figure 3.2). This within-species variation was also observed by other studies looking at SCC by SaM species, including Fry et al. (2014) and Supré et al. (2011). Quarters with an IMI due to *S. chromogenes* had an especially wide span of observed quarter SCC in the current study, ranging from 2,000 (the lower limit of detection) to 6,100,000 cells/mL. This variability in the effect of *S. chromogenes* on quarter SCC was also noted in Valckenier et al. (2021), where quarters classified as having a transient IMI due to *S. chromogenes* had a mean SCC of 69,000 cells/mL, while those classified as having a persistent *S. chromogenes* IMI had an 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 no growth (≤ 50,000 cells/mL) and infected, but with no observable clinical signs (> 50,000 cells/mL), as well as one of the three most common species in quarters exhibiting clinical signs of mastitis. Similarly, Condas et al. (2017b) found that in NASM-positive quarters, *S. chromogenes* was isolated with similar frequency from quarters classified as low-SCC (< 200,000 cells/mL), high SCC (> 200,000 cells/mL), and those with clinical mastitis. This observed diversity in the effect of *S. chromogenes* may suggest that strain type could play a role in the variable pathogenicity of NASM species, as some previous work suggests (Hyvönen et al., 2009; Åvall-Jääskeläinen et al., 2013; Naushad et al., 2019). More work exploring the possible effect of strain type while accounting for cow-level effects (i.e., immune response, DIM, parity), especially for *S. chromogenes*, is warranted to further understand this variability of observed effect on quarter SCC. As we further understand the ecology and epidemiology of individual NASM species and identify species or strains with host-adapted or contagious behavior, speciation and strain typing for NASM will be important as a part of mastitis control decision making.

A large amount of variability was also seen in the observed qSCC for no growth quarters (as defined by bacteriological status), which ranged from 2,000 (lower limit of detection) to 8,400,000 cells/mL. The presence of some relatively high quarter SCC observations in this group highlights the limitation of using culture as a method for identifying the quarter IMI status, as was recognized by Fry et al. (2014). Researchers in that study point out that the low sensitivity of bacterial culture as a test for IMI may have resulted in the presence of some undiagnosed IMI in the quarters defined as no growth. For a quarter to be considered culture negative in the current study, both milk samples were required to have either no growth at all or no significant growth on both plates. Despite the imperfect nature of bacteriological culture for determining IMI status, the median (Figure 3.2) and mean (Table 3.2) SCC for the negative control quarters was still well below that of most SaM species.

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

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

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

3.6 Conclusions

The current study describes the species-specific effect of intramammary infection with staphylococci and mammaliicocci on quarter somatic cell count for a population of organic dairies. The diversity of SaM species observed on these 10 organic dairy herds and the species-level effect on qSCC was similar to previous studies in conventional herds. *S. chromogenes* was the most frequently found species, followed by *S. aureus, S. haemolyticus,* and *S. simulans.* Compared to culture no growth quarters, qSCC was higher in quarters infected with 9 of 10 SaM species identified. The highest cell count was for quarters infected with *S. warneri,* followed by *S. aureus, S. agnetis,* and *S. hyicus.* A large amount of variability was observed in qSCC for quarters infected with *S. chromogenes*, *S.* *haemolyticus, S. simulans,* and *S. aureus.* Although the increase in qSCC was modest for most SaM species observed, the widespread nature of these intramammary pathogens can still result in sizeable increases in bulk tank SCC.

3.7 Notes

This project was funded by the Organic Agriculture Research and Extension Initiative (OREI) from the National Institute of Food and Agriculture (USDA-NIFA grant 2018-51300-28561). The first author, Caitlin E. Jeffrey was supported by a USDA-NIFA Predoctoral Fellowship award (grant 2022-67011-36567). The authors thank the organic dairy producers who agreed to participate in this study, for giving us their time and allowing us to collect samples from their farms. We are grateful to the numerous University of Vermont undergraduate students who assisted with sample collection. We thank the laboratory staff at the Vermont State Agricultural and Environment Laboratory for determination of somatic cell counts from the quarter milk samples. We thank Paige Isensee, Natalie Sexton, Madyson Marrs, and Allena Radford, who provided laboratory assistance in the Adkins lab at the University of Missouri. Caitlin Jeffrey conceptualized the study, acquired funding, coordinated farm recruitment and sampling, conducted on-farm sample collection, managed and curated the data, conducted the data analysis, prepared data visualizations and presentation, wrote the original and final drafts. Pamela Adkins, conducted isolate species identification by MALDI-TOF, reviewed and edited the manuscript. Simon Dufour led statistical analysis and use of techniques for analysis and synthesis of the study data, reviewed and edited the manuscript. John Barlow conceptualized the study, acquired funding, supervised the research, conducted on-farm sample collection, reviewed and edited the manuscript. The authors have not stated any conflicts of interest.

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3.9 Tables

|  |  |  |  |
| --- | --- | --- | --- |
| Table 3.1Final multivariable model describing the effect of intramammary infection with frequently isolated staphylococci and mammaliicocci on quarter somatic cell score, adjusted for days in milk at time of sampling. Data set is comprised of 2,620 quarter-day observations collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). | | | |
| *Fixed effects* | | | |
| Quarter-day IMI status | No. quarter observations | Coefficient estimate (SE) | *P*-value |
| Intercept | - | -0.03 (0.29) | 0.90 |
| *S. warneri\** | 15 | 5.18 (0.60) | < 0.001 |
| *S. aureus\** | 112 | 4.81 (0.22) | < 0.001 |
| *S. agnetis\** | 21 | 3.76 (0.45) | < 0.001 |
| *S. hyicus\** | 6 | 3.23 (0.85) | < 0.001 |
| *S. simulans\** | 35 | 3.11 (0.39) | < 0.001 |
| *S. xylosus\** | 11 | 2.96 (0.62) | < 0.001 |
| *S. chromogenes\** | 384 | 2.88 (0.12) | < 0.001 |
| *S. haemolyticus\** | 40 | 1.77 (0.31) | < 0.001 |
| *S. devriesei\** | 15 | 1.62 (0.54) | 0.003 |
| *S. equorum* | 9 | 0.12 (0.48) | 0.81 |
| No growth | 1972 | *Reference* | *Reference* |
| Days in milk | - | -0.003 (0.01) | 0.54 |
| Days in milk2 | - | < 0.001 (< 0.001) | 0.73 |
| Days in milk3 | - | < 0.001 (< 0.001) | 0.53 |
| *Random effects* | Variance |  | |
| Farm | 0.28 |  | |
| Cow | 1.0 |  | |
| Quarter | 0.47 |  | |
| \* Quarter somatic cell score differs from no growth quarters (*P* ≤ 0.05) | | | |

|  |  |  |
| --- | --- | --- |
| Table 3.2 Estimated quarter somatic cell count by intramammary infection status at 91 days in milk (13 weeks) for frequently isolated staphylococci and mammaliicocci and no growth quarters. Data set used to make model estimations is comprised of 2,620 quarter-day observations collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). | | |
| Quarter-day IMI status | Estimated quarter somatic cell count (cells/mL) | 95% lower and upper confidence level (cells/mL) |
| *S. warneri* | 395,190 | 148,189 - 1,053,891 |
| *S. aureus* | 307,101 | 197,323 - 477,951 |
| *S. agnetis* | 148,437 | 69,021 - 319,232 |
| *S. hyicus* | 102,478 | 26,368 - 398,281 |
| *S. simulans* | 94,617 | 48,346 - 185,175 |
| *S. xylosus* | 84,985 | 30,798 - 234,512 |
| *S. chromogenes* | 80,376 | 56,942 - 113,454 |
| *S. haemolyticus* | 37,333 | 21,217 - 65,688 |
| *S. devriesei* | 33,513 | 13,597 - 82,599 |
| *S. equorum* | 11,855 | 5,292 - 26,556 |
| No growth | 10,927 | 8,056 - 14,822 |

3.10 Figures

A diagram of a flowchart

Description automatically generated

Figure 3.1Flow diagram describing selection of final data set of quarter-day observations collected from 382 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US).



Figure 3.2Somatic cell score for 2,260 quarter-day observations with an intramammary infection due to staphylococci and mammaliicocci and no growth quarters. Quarter-day observations were collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). The red dotted line indicates a somatic cell score of 4. The observed data are displayed (i.e., quarters that were repeatedly positive for the same species contributed several SCC measurements). Each box contains 50% of the data for a species, the median (line), and is bounded by the 25th and 75th percentiles. The upper whisker represents the largest observation less than or equal to the 75th quartile plus 1.5 times the interquartile range, while the lower whisker represents the smallest observation greater than or equal to the 25th quartile minus 1.5 times the interquartile range.



Figure 3.3 Quarter somatic cell score least square means estimates as a function of staphylococci and mammaliicocci IMI and days in milk, compared to no growth quarters. Data set used to make model estimations is comprised of 2,620 quarter-day observations collected from 1,272 quarters belonging to 360 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). Model estimates for each species are only presented for the range of days in milk for IMI observations in the data set. Error bars represent the 95% confidence interval.

CHAPTER 4: Antimicrobial resistance genes, virulence potential, and strain type of Staphylococcus chromogenes causing bovine intramammary infections with low vs. high somatic cell counts

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4.1 Abstract

*Staphylococcus chromogenes* is the dominant species associated with mammary gland infections in dairy animals and one of the most persistent intramammary pathogens*.* The objectives of the current study were to: 1) identify if distinct strain types (ST) of *S. chromogenes* were associated with persistent intramammary infections (IMI) where quarter somatic cell count (SCC) is consistently elevated (HIGH SCC IMI) vs. consistently low (LOW SCC IMI), 2) identify if *S. chromogenes* from persistent HIGH SCC IMI are more likely to carry antimicrobial resistance genes (ARGs) vs. LOW SCC IMI, and 3) identify if *S. chromogenes* from persistent HIGH SCC IMI possess more genes encoding previously-described staphylococcal virulence factors (VF) vs. LOW SCC IMI. Isolates originate from a longitudinal, observational study of 10 organic dairies in Vermont (US), where aerobic culture of quarter-milk samples to identify IMI was conducted in parallel with determination of quarter SCC. Two groups were selected from persistent (≥ 30 days) *S. chromogenes* IMI (as confirmed by RAPD-PCR): 1) IMI associated with high SCC, where all quarter-day observations had an SCC of ≥200,000 cells/mL; and 2) IMI associated with low SCC, where all quarter-day observations had an SCC of <200,000 cells/mL. Representative isolates from 15 LOW SCC IMI and 15 isolates from HIGH SCC IMI were submitted for whole genome sequencing and strain-typed according to a 7-locus MLST scheme*.* ARG and VF were identified from assembled genomes. Separate mixed-effects logistic regression models were made using ST, ARG carriage, and VF number as the predictor, SCC category as the outcome, with herd and cow as random effects. Ten different ST were identified, including 4 novel ST. Seven ST were identified in each SCC category, with 3 unique to each. In a mixed-effects logistic regression, ST was not a predictor of SCC category. The only ARG identified was *blaZ,* encoding for resistance to penicillin (33.3% of isolates; 6/15 in the HIGH SCC category and 4/15 in the LOW SCC category). *blaZ* was not a predictor of SCC category in a mixed-effects logistic regression model. *blaZ* was consistently present in all isolates for 4/5 ST with multiple isolates. Sixty-two unique VF were identified (median: 44 per isolate; range: 43-51). Thirty-nine VF were present in all isolates, including genes associated with iron uptake and metabolism, production of phenol-soluble modulins, hemolysins, and an exfoliative toxin. Presence of VF associated with adherence, host immune evasion, type VII secretion system, and production of exoenzymes and exotoxins varied among isolates. In the HIGH SCC category, 677 VF total were identified vs. 670 in the LOW SCC category. In a mixed-effects logistic regression, number of VF identified was not a predictor of SCC category. Genes encoding for exfoliative toxin type C (*etc*) and staphylocoagulase (*coa*) were identified in isolates from the current study, neither of which have been widely reported for *S. chromogenes* isolates of bovine origin. *blaZ* carriage, number and type of VF appear to be a function of ST for *S. chromogenes*, but more research is needed to confirm these findings.

4.2 Introduction

*Staphylococcus chromogenes* is the leading cause of intramammary infections (IMI) in dairy cattle worldwide, for both conventional (De Visscher et al., 2016; Condas et al., 2017a; Rowe et al., 2019; Wuytack et al., 2020a) and organic (Peña-Mosca et al., 2023) herds in various countries. *S. chromogenes* is categorized as belonging to a heterogeneous group of bacteria known as the non-*aureus* staphylococci (NAS),although species within this group exhibit varying pathogenicity when causing IMI. Within NAS, *S. chromogenes* is of special concern due to its ability to be both persistent and cause an inflammatory reaction increasing quarter somatic cell count (SCC) (Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014a), even to the point where the SCC of quarters infected with *S. chromogenes* were no different than quarters infected with a major mastitis pathogen such as *S. aureus* (Wuytack et al., 2020a; Valckenier et al., 2021; Woudstra et al., 2023).

Beyond the marked differences between NAS, variation in pathogenicity has also been demonstrated for different strains within the same species. Intraspecies variation has been observed in effect on SCC (Supré et al., 2011; Fry et al., 2014a; Condas et al., 2017a), differences in interaction with host immune cells (Hyvönen et al., 2009; Åvall-Jääskeläinen et al., 2013), persistence of infection (Mork et al., 2012; Valckenier et al., 2021), and effect on milk production (Thorberg et al., 2009). For *S. chromogenes* specifically, heterogeneity exists in populations of isolates causing IMI. In a study of 8 Belgian herds, Wuytack et al. (2020a) identified *S. chromogenes* as one of the most prevalent NAS species causing IMI in quarters with various infection phenotypes. *S. chromogenes* was the leading cause of IMI in quarters identified as “healthy” (SCC of ≤ 50,000 cells/mL), as well as those identified as infected with no observable clinical signs (SCC of > 50,000 cells/mL; Wuyatack et al., 2020a). Moreover, *S. chromogenes* ranked among the top 3 species in quarters exhibiting clinical mastitis (Wuytack et al., 2020a). Similarly, Condas et al. (2017b) found that among 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. Different strains of *S. chromogenes* vary in their interaction with host immune cells and inflammatory response (Breyne et al., 2015; Piccart et al., 2016; Souza et al., 2016), as well as frequency of isolation from different habitats or sources (skin vs. mammary gland; Wuytack et al., 2020b).

An association has also been demonstrated between different traits related to clinical signs or pathogenicity for staphylococcicausing IMI*.* Valckenier et al. (2021) describe a link between persistence of infection and associated SCC, 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 mean SCC of 351,000 cells/mL. Wuytack et. al (2020a) used PCR to screen for the presence of genes encoding staphylococcal virulence factors (VF) in 59 NAS isolates from IMI, and then compared the number of VF detected in isolates originating from quarters classified into 2 different infection phenotypes: quarters with clinical signs, versus quarters with an SCC of ≤ 50,000 cells/mL. Of the 17 VF screened for, 4 were detected among NAS isolates included in the study: *cap5H,* a gene associated with capsule formation; *bap,* a gene associated with biofilm formation; *agrA,* a transcription factor associated with the production of virulence factors and hemolysins; and *mecA,* a gene conferring resistance to methicillin. Only 25% of isolates from low SCC quarters were found to be positive for any of these 4 VF (3 isolates positive for *bap* only, 1 positive for *mecA* only), while 72% of isolates from quarters with clinical signs were positive for at least 1 of the 4 VF (Wuytack et al., 2020a).

In a study by Haveri et al. (2005) of 217 *S. aureus* IMI isolates typed using pulsed-field gel electrophoresis (PFGE), researchers were able to identify that a particular pulsotype was significantly associated with severe clinical mastitis symptoms but reduced persistence when compared to the 4 other commonly identified pulsotypes in the study. This association between a specific genotype and consistent expression of a clinical trait associated with an IMI has not yet been widely described for NAS. However, researchers in a large Canadian study investigating the profile of staphylococcal VF for 25 different species of NAS identified 2 rather distinct populations among the 83 *S. chromogenes* included (Naushad et al., 2019). In a cluster analysis looking at the distribution of all 191 VF for the 441 genomes of isolates included in the study, *S. chromogenes* was the only species split into 2 distinct populations: the majority of *S. chromogenes* isolates clustered together with a profile distinct to their species, but a small number clustered with isolates belonging to other closely related species (Naushad et al., 2019). The authors point out this may be a result of including a larger number of *S. chromogenes* isolates compared with other species, but also suggest this finding could represent separate pathotypes of *S. chromogenes* causing bovine IMI. Missing from these analyses are replicable sequence-based typing results that would better facilitate comparison of the findings from Naushad et al. (2019) with later studies. Subsequent to this publication, a multilocus sequence typing (MLST) scheme was published to fill that void (Huebner et al., 2021).

In a longitudinal study of 10 certified organic dairy farms in Vermont (US), *S. chromogenes* was the most common pathogen causing subclinical mastitis (Jeffrey et al., unpublished manuscript). In agreement with the heterogeneity observed in Wuytack et al. (2020a) and Condas et al. (2017b), the qSCC associated with *S. chromogenes* IMI in our study ranged from 2,000 cells/mL (the lower limit of detection) to 6,100,000 cells/mL (Jeffrey et al., unpublished manuscript). Furthermore, 72% of *S. chromogenes* IMI persisted for at least 60-90 days during the study period (Jeffrey et al., unpublished manuscript). The aim of the current study is to better understand the diversity within *S. chromogenes* causing bovine IMI by identifying if there is a genetic basis for the observed difference in pathogenicity (i.e., the infection phenotype, as measured by qSCC). The specific objectives were to: 1) identify if distinct strain types (ST) of *S. chromogenes* are associated with persistent IMI where qSCC is consistently elevated (HIGH SCC IMI) vs. consistently low (LOW SCC IMI), 2) identify if *S. chromogenes* from persistent HIGH SCC IMI are more likely to carry antimicrobial resistance genes (ARGs, as determined by whole genome sequencing) vs. LOW SCC IMI, and 3) identify if *S. chromogenes* from persistent HIGH SCC IMI possess more genes encoding previously-described staphylococcal VF vs. LOW SCC IMI. The null hypotheses for this study were that: 1) there would be no association between ST and qSCC category of isolates, 2) there would be no difference in frequency of ARGs between isolates in the 2 SCC phenotype groups, and 3) there would be no difference in frequency of VF genes between isolates in the 2 SCC phenotype groups.

4.3 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).

4.3.1 Sample origination

Isolates included in the current study originated from milk samples collected during a longitudinal observational study of 10 certified organic dairy farms in Vermont (US) conducted 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. The inclusive term “bedded pack” is used here to encompass both aerobically composting bedded packs and deep bedded packs, and was defined as an enclosed loose housing facility deeply bedded with organic material (Jeffrey et al., 2024). 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; or Dairy One Co-Op. Inc., Ithaca, NY) to obtain calving dates and parity for individual lactating cows. Calving dates and parity for 1 herd was obtained from personal communication with the producer who kept written records. The goal was to enroll 35 early- to mid-lactation cows stratified by parity, current SCC, and DIM from each herd to sample repeatedly for the duration of the study. In 1 herd with approximately 35 lactating cows, all cows were enrolled. 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 10 x 10 cm 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. If any sign of clinical mastitis was present, it was noted, and that sample was excluded from inclusion in this study. 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 at -20°C for SCC measurement and the remaining milk sample was processed for bacteriological culture.

4.3.2 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). It has been demonstrated that freezer storage and gradual thawing of milk samples has only a limited impact on SCC measurements (Barkema et al., 1997), and previously published work has relied on measuring SCC from frozen quarter milk samples for determining the extent of inflammation associated with NAS IMIs (Fry et al., 2014a).

4.3.3 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 homogenizing milk samples 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).

4.3.4 Identification of bacterial isolates to species

Isolates from both pure and mixed culture quarter-milk samples were selected and grown in pure culture on blood agar. Standard benchtop tests were performed 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 (Haw et al., 2024). 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 non-*aureus* staphylococci and mammaliicocci species identification were applied as previously described (Cameron et al., 2017), in which ≥1.7 was used for species-level identification and <1.7 was classified as inconclusive. Suspect staphylococci and mammaliicocci isolates unable to be identified to the species level and those identified as *Staphylococcus agnetis* or *Staphylococcus hyicus* by MALDI-TOF were speciated using *tuf* gene sequences with a cut-off of 98% identity as previously described (Hwang et al., 2011).

4.3.5 Determination of IMI status and selection of persistent IMI

Using the bacteriological status and speciation information, a quarter-day IMI status was assigned to each quarter observation: 1) “no growth,” when there was no significant growth; 2) “infected with *S. chromogenes* only,” when ≥100 CFU/mL of *S. chromogenes* was identified in pure culture on both plates (interpretation in series; Dohoo et al., 2011); 3) “mixed infection with *S. chromogenes*,” when ≥100 CFU/mL of *S. chromogenes* and an additional species were identified in mixed culture on both plates; 4) “infected with pathogen other than *S. chromogenes*,” when ≥100 CFU/mL of a species besides *S. chromogenes* was identified in pure or mixed culture on both plates; and 5) “unknown” if the sample status had been identified as contaminated or indeterminate as previously described. Quarter-day observations were eligible for inclusion in further analysis if they had an associated quarter-level SCC measurement and the IMI status was classified as infected with *S. chromogenes* only.

A given quarter was considered to have a potentially persistent *S. chromogenes* IMI if: 1) it had ≥ 2 quarter-day observations (from sequential sampling events approximately 30 days apart); 2) IMI status could be determined for all sampling events associated with that quarter; and 3) it was infected with *S. chromogenes* only for all associated quarter-day observations throughout the study. Quarter-day observations of *S. chromogenes* IMI in a mixed infection with another species were not eligible for inclusion, as the impact of the other pathogen on qSCC could not be isolated from the effect of *S. chromogenes*. Two groups were then selected from all potentially persistent *S. chromogenes* IMI: 1) IMI associated with high SCC, where all quarter-day observations had an associated SCC of ≥200,000 cells/mL; and 2) IMI associated with low SCC, where all quarter-day observations had an associated SCC of <200,000 cells/mL. Although various other SCC schemes have been used to categorize the inflammation severity associated with mastitis in a quarter (Naushad et al., 2019; Wuytack et al., 2020a), a qSCC of 200,000 cells/mL was chosen for the current study. This cut-off is a well-established threshold most indicative of an increased likelihood of subclinical intramammary infection (Schukken et al., 2003) and has previously been used to categorize quarters by infection phenotypes (Condas et al., 2017). Any potentially persistent *S. chromogenes* IMI that did not fit into 1 of these 2 categories was excluded from further analysis (e.g., persistent IMI that had an SCC of <200,000 cells/mL for one or more quarter-day observations and an SCC of ≥200,000 cells/mL for any other observation in that quarter were not included).

4.3.6 Strain-typing and selection of isolates

All isolates associated with each potentially persistent high and low SCC *S. chromogenes* IMI were strain-typed using random amplification of polymorphic DNA (RAPD)-PCR. DNA was extracted using a commercial kit from overnight broth culture following the manufacturer’s instructions (DNeasy Blood and Tissue Kit, Qiagen) and then stored at -20 °C until further analysis. RAPD-PCR was performed as described by Wuytack et al. (2020b) using the primer set D11344 (Fitzgerald et al., 1997) with the following PCR conditions: 4 cycles of 94 °C at 5 min, 36 °C at 5 min, and 72 °C at 5 min and 30 cycles of 94 °C at 1 min, 36 °C at 1 min, and 72 °C at 2 min. A negative control (no DNA) was included for each amplification. Amplified DNA fragments were separated on 1.5% (wt/vol) agarose gels stained with SYBR Safe (0.1 µL/mL; ThermoFisher Scientific) at 120 V for 75 min, and then photographed by UV transillumination (Image Lab, Bio-Rad). The RAPD-PCR product of all isolates from a given persistent IMI within a quarter were analyzed in the same PCR amplification and were run in adjacent lanes on the same gel. The images were inspected visually, and isolates with the same banding pattern, number, and size of bands were considered to be the same RAPD type. If theisolates from all quarter observations of an *S. chromogenes* IMI belonged to the same RAPD type, the quarter was considered persistently infected with the same strain.

In order to describe the diversity of *S. chromogenes* RAPD types among persistent IMI within each herd, 1 representative isolate was selected from each confirmed persistent IMI for strain comparison. The RAPD-PCR products from all representative isolates within a herd were run in adjacent lanes on a gel and imaged (as described above) along with a 1 kb bp ladder for image standardization. Gel images were imported into BioNumerics version 7.5 (AppliedMaths, Sint-Martens-Latem, Belgium) and analyzed using the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) with both optimization and position tolerance set at 1.0%. Isolates from the same herd with 100% similarly were considered the same RAPD type.

From among the confirmed persistent *S. chromogenes* IMI, 15 quarters with a persistently low SCC IMI (LOW SCC IMI) were selected to match the 15 quarters with a persistently high SCC IMI (HIGH SCC IMI). A priori sample size calculations were not performed because only 15 high SCC IMI were identified. As a result, we were limited to selecting 15 IMI from each of the 2 SCC categories. LOW SCC IMI quarters were matched to HIGH SCC IMI quarters belonging to the same cow (different quarter) when possible. If this was not possible, LOW and HIGH SCC IMI quarters were matched on farm, or facility type (bedded pack vs. tiestall) when same farm was not possible. When LOW and HIGH SCC IMI quarters were paired between different cows, quarters were matched as closely as possible to ensure a similar DIM and parity. From each of the 15 HIGH and 15 LOW SCC IMI, a representative isolate was chosen to undergo whole genome sequencing (WGS). For each persistent IMI within a quarter that had the same RAPD type across 3 quarter-day observations, the middle isolate in the series was submitted for WGS. For persistent IMI within a quarter that had the same RAPD type across 2 quarter-day observations, 1 of the 2 isolates in the series was arbitrarily selected for WGS.

4.3.7 DNA extraction, whole genome sequencing, assembly, and annotation

Each of the 30 *S. chromogenes* isolates selected for WGS were recovered from frozen stock on a tryptic soy agar with 5% sheep blood (TSA) plate incubated under aerobic conditions at 37°C, and read at approximately 24 and 48 hrs. All plates were then inspected to ensure purity, and a single colony was selected and passed to a new TSA plate. After again being incubated at 37°C, read at approximately 24 and 48 hrs, and checked for contamination, 48-hr growth plates were wrapped in Parafilm (Amcor). Wrapped plates were sent overnight to a commercial sequencing facility (SeqCoast Genomics; Portsmouth, NH, USA) for DNA extraction, library preparation, long read sequencing using GridION Oxford Nanopore, paired-end sequencing using Illumina, assembly, and annotation. DNA extraction was performed on colony material collected from the agar plates with a commercial kit using bead beating lysis (MagMAX Microbiome Ultra Nucleic Acid Isolation Kit, Applied Biosystems). Library preparation was completed using Illumina DNA Prep tagmentation kit (Illumina), and paired-end sequencing (2x150bp) was run on the Illumina NextSeq2000 platform (Illumina). During Illumina sequencing, 1-2% PhiX control was spiked into the run to support optimal base calling, and read demultiplexing, read trimming, and run analytics were performed on the instrument using DRAGEN v3.10.12. Library preparation for long-read sequencing was completed using the Oxford Nanopore Technologies SQK-LSK114 native barcoding kit, and sequencing was performed on the GridION platform (FLOW-MIN114 Spot-ON Flow Cell, vR10). Quality-trimming of raw reads was completed using Trimmomatic v0.39 (Bolger et al., 2014) and Porechop v.0.2.4 (https://github.com/rrwick/Porechop) for reads from Illumina and Oxford Nanopore sequencing, respectively. Unicycler v0.4.4 (Wick et al., 2017) was used for hybrid assembly of all genomes. Briefly, the trimmed Illumina reads were assembled using SPAdes v3.14.0 (Bankevich et al., 2012) and then mapped with trimmed error-corrected Oxford Nanopore reads using Bowtie2 (Langmead and Salzberg, 2012) and SAMtools (Li et al., 2009). The polishing of the final hybrid assembly was done using Pilon (Walker et al., 2014), and annotation was completed using BAKTA v1.5.1 (Schwengers et al., 2021).

4.3.8 Bioinformatic analyses, in silico MLST, and detection of ARG and VF

MLST were predicted *in silico* from the annotated genomes for the 7-locus scheme described for *S. chromogenes* (Huebner et al., 2021) using the MLST 2.0 tool (Center for Genomic Epidemiology, Technical University of Denmark, Kongens Lyngby, Denmark; software v2.0.9, database v2023-06-19; MLST allele sequence and profile data obtained from PubMLST.org). Any novel alleles identified were confirmed using traditional PCR and Sanger sequencing by dye of amplicons in both directions at the University of Vermont Genomics Core Facility following the methods of Huebner et al. (2021). The reverse and forward chromatograms were aligned and screened for quality using Geneious Prime® software (version 2023.1.2, Biomatters Ltd.). Novel alleles or allelic profiles were submitted to the MLST database curator for new allele and ST number assignment. All isolates from the current study were submitted to the database (https://pubmlst.org/organisms/staphylococcus-chromogenes).

The 7-locus concatenated nucleotide sequence data were then combined with all 386 concatenated MLST sequences for *S. chromogenes* available in PubMLST database as of July 25th, 2024. The resulting FASTA file was used for the construction of a phylogenetic tree by maximum-likelihood algorithm with the optimal model and 100 bootstrap replications in MEGA-X (Kumar et al., 2018). Isolates which grouped together with a bootstrap value of ≥ 65% were classified as clusters.

ARG were identified from assembled genomes using ABRicate v1.01, which draws from 5 different databases [ResFinder from Center for Genomic Epidemiology (Camacho et al., 2009; Bortolaia et al., 2020), Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020), MegaRES v3.0 (Bonin et al., 2023), ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation), and AMRFinderPlus from NCBI (Feldgarden et al., 2021)] using the default settings (https://github.com/tseemann/abricate). VF were identified from assembled genomes using the VFDB tool (Chen et al., 2016) and a “blastp” search against a published comprehensive dataset of staphylococcal VF (Naushad et al., 2019). After the blast search, the best hit of virulence genes for each genome was chosen based on *H* values, as described by Naushad et al. (2019). Briefly, an *H* value was calculated to determine homology between query protein sequences and blast hits (Fukiya et al., 2004). *H* values (in units of amino acids) between protein sequences were calculated using the following formula: *H* = *VFid × Lm/Lq*, where *VFid* represents the percent similarity between the VF query sequence and the identified protein sequence (expressed as proportion between 0 and 1), where *Lm* represents the alignment length, and *Lq* denotes the length of the query sequence (Fukiya et al., 2004). A cutoff was established for sequence similarity of 30% and a query length coverage of 50%, with any hits having values below these cutoffs discarded from the data set. Hits from each query sequence were then sorted according to their *H* value, and the hit with the largest *H* value (highest sequence similarity and query length coverage) was selected in order to prevent 1 VF query returning hits to 2 different genes within a given genome. The list of remaining VF were classified into 5 functional categories, as outlined in Naushad et al. (2019): 1) adherence, 2) exoenzymes, 3) host immune evasion, 4) iron uptake and metabolism, and 5) toxins (including hemolysins, leukocidins, leukotoxins, toxic shock syndrome toxin, exfoliative toxins, type VII secretion system genes, phenol-soluble modulins, enterotoxins, and exotoxins).

4.3.9 Statistical analysis

A spreadsheet (Microsoft Excel, Redmond, WA) with isolate identification, associated metadata, and outcome variables was created and imported into the R Statistical Programming Environment (R Development Core Team, 2023) for analysis. Descriptive statistics were generated to compare parity and DIM of the cow, quarter location, and average SCC associated with each persistent IMI between the two SCC categories (HIGH vs. LOW). Normality of the data was checked using a Shapiro test. For outcomes which were not normally distributed (parity, DIM, average SCC), a Mann Whitney U test was used to compare metrics between the HIGH and LOW SCC IMI groups. For outcomes which were normally distributed (quarter location), Fisher’s Exact test was used to compare the two groups. Statistical significance for these tests were declared at *P* ≤ 0.05.

Separate mixed-effects logistic regression models were made using ST, ST cluster, *blaZ* carriage, and VF number as the predictor, SCC category as the outcome, and both herd and cow as random effects using the “lme4” package (R Development Core Team, 2023). The variable representing the number of VF genes identified per isolate was centered and scaled by subtracting the mean and dividing by the standard deviation. Significance of predictors was assessed using a cutoff of ≤0.05 for the *P-*value associated with the z-statistic.

4.3.10 Data availability

The raw reads from ONT and Illumina for all 30 genomes are available under NCBI Bioproject accession number PRJNA1130504 (Biosamples SAMN42232476 to SAMN42232505) in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/). Isolates from the current study are isolate number 405 to 434 in the PubMLST *S. chromogenes* database (https://pubmlst.org/organisms/staphylococcus-chromogenes).

4.4 Results

4.4.1 Descriptive results, MLST and phylogenetic analyses

In total, 190 *S. chromogenes* IMI were identified from the 10 herds, including 137 which met the previously described criteria for a potentially persistent *S. chromogenes* IMI. One *S. chromogenes* IMI was classified as a clinical mastitis event. This quarter was identified as having an IMI due to *S. chromogenes* for the first quarter-day observation, and no growth on the second quarter-day observation. There were no clinical mastitis observations among the potentially persistent IMI. There were 92 potentially persistent IMI which were associated with 3 sequential quarter-observations and 45 which were associated with 2. There were 15 potentially persistent IMI for which all quarter-day observations had an SCC of ≥200,000 cells/mL, 61 for which all quarter-day observations had an SCC of <200,000 cells/mL, 61 for which the SCC was both above and below 200,000 cells/mL. Of the 61 LOW SCC IMI, 46 were associated with 3 sequential quarter-observations (138 isolates), and 15 were associated with 2 sequential quarter-observations (30 isolates). Of the 15 HIGH SCC IMI, 3 were associated with 3 sequential quarter-observations (9 isolates), and 12 were associated with 2 sequential quarter-observations (24 isolates). One hundred and ninety-eight isolates associated with 75 potentially persistent *S. chromogenes* IMI underwent RAPD-typing, with 74 of the 75 IMI determined to be caused by the same strain (Figure 4.1). One LOW SCC IMI included an isolate which was not identified to be *S. chromogenes* until after RAPD-typing had been completed, so was not included in the current study. The median number of persistently high and low SCC IMI per herd was 8 (Table 4.1; range: 3-14), and the median number of RAPD types associated with these IMI was 5 (range: 2-9).

A total of 30 representative isolates (15 from HIGH and 15 from LOW SCC IMI) were selected for WGS. Parity group (first, second, third, fourth and above), DIM, and quarter position did not differ between the HIGH and LOW SCC IMI group (*P* = 0.88, 0.14, 0.88, respectively). These 30 representative isolates originated from 7 of the sampled organic herds, with 16 isolates derived from herds using a bedded pack facility and 14 isolates from herds using a tiestall facility (Table 4.2). The 30 IMI were from 25 different cows, with 3 cows contributing 2 quarters each and 1 cow contributing 3 quarters. Thirteen IMI were associated with 3 sequential quarter-observations and 17 were associated with 2 sequential quarter-observations. Isolates in the HIGH SCC IMI group were from 6 different farms (n = 8 bedded packs and 7 tiestalls), while isolates in the LOW SCC IMI group also came from 6 different farms (n = 8 bedded packs and 7 tiestalls). The median parity and DIM of the cows from which the isolates originated was 2 (range: 1-6) and 281 days (range: 58-438 days) for the HIGH SCC IMI group, and 2 (range: 1-6) and 229 days (range: 41-438 days) for the LOW SCC IMI group, respectively. The median of the mean SCC associated with each IMI was 410,000 cells/mL (range: 230,000-2,798,000 cells/mL) for the HIGH SCC IMI group, and 98,500 cells/mL (range: 28,000-185,000 cells/mL) for the LOW SCC IMI group. The mean SCC associated with each IMI in the HIGH SCC group was greater than that of the LOW SCC group (*P* <0.001).

Ten different MLST were identified among the 30 representative isolates which underwent WGS, with 7 ST identified in each the HIGH and LOW SCC IMI categories (Table 4.3). Four novel ST were identified which were not already present in the PubMLST database for *S. chromogenes* (ST174 through ST177). Four ST were found in both SCC categories (ST5, ST6, ST48, ST176), 3 were unique to the HIGH SCC IMI category (ST25, ST136, ST177), and 3 were unique to the LOW SCC IMI category (ST51, ST174, ST175). The most common ST identified were ST6 and ST176, with 18 isolates (60%) belonging to 1 of these 2 ST (9 isolates, or 30%, belonging to each ST6 and ST176 respectively). In a dendrogram constructed from concatenated nucleotide sequence data for the study isolates in combination with 386 publicly available concatenated MLST sequences for *S. chromogenes*, 5 ST clusters were identified where study isolates which grouped together with a bootstrap value of ≥ 65% (Supplemental Figure S1). Ninety percent of isolates (27/30) were able to be assigned to 1 of these 5 ST clusters. The 3 remaining isolates were the only example of their respective ST.

4.4.2 Analysis of associations between ST (or ST cluster) and SCC category

In a mixed-effects logistic regression, ST was not found to be a significant predictor of whether an isolate would be in the HIGH or LOW SCC IMI category (*P* <0.05). As 5 isolates were singleton ST, and the 2 isolates belonging to ST25 were both in the HIGH SCC IMI category, this model was run for a dataset containing the remaining 23 isolates (belonging to 4 different ST: 5, 6, 48, 176). Similarly, ST cluster was not found to be a significant predictor of whether an isolate would be in the HIGH or LOW SCC category (*P* <0.05) for a dataset containing the 25 isolates able to be grouped into 1 of the 4 ST clusters identified with isolates in both SCC categories (ST clusters 48, 5, 6, and 1). Three separate models (with all 30 isolates in the dataset for each) were run to see if belonging to ST176, ST cluster 1, or ST6 predicted the SCC category of an isolate. However, all three models found that belonging to each of these 3 groupings was not a significant predictor of SCC category (*P* = 0.75 for ST176; *P* = 0.33 for ST1 cluster; *P* = 0.86 for ST6).

4.4.3 Antimicrobial resistance genes and associations between ARG and SCC category

The only resistance gene identified among the 30 *S. chromogenes* isolates was *blaZ*. Ten of the 30 (33%) *S. chromogenes* isolates were positive for *blaZ,* 6/15 (40%) in the HIGH SCC IMI category and 4/15 (26.7%) in the LOW SCC IMI category (Figure 4.2). *blaZ* gene carriage was not found to be a significant predictor of SCC category (HIGH vs. LOW) for an isolate in a mixed-effects logistic regression model (*P* = 0.50). As *blaZ* carriage was consistently present in all isolates for 4 of the 5 ST with multiple isolates (Table 4.4), statistical analysis exploring if ST predicted *blaZ* carriage was not possible. All isolates belonging to ST5 (n=3), ST48 (n=2), and ST51 (n=1) were *blaZ* positive, while no isolates belonging to ST25 (n=2) and ST176 (n=9) carried the gene. Only isolates belonging to ST6 varied in *blaZ* carriage (4 of 9 isolates were positive).

4.4.4 Virulence genes identified and analysis of associations between VF and SCC category

There were 62 different VF detected among the 30 *S. chromogenes* isolates (Table 4.5). There were 39 VF identified which were present in 100% of isolates (Figure 4.3), which included all genes associated with iron uptake and metabolism, and those associated with production of phenol-soluble modulins, hemolysins, and an exfoliative toxin. Presence of VF associated with adherence, host immune evasion, type VII secretion system, and production of exoenzymes and exotoxins varied between isolates. Some patterns of presence or absence of VF was specific to particular ST. This included the presence of *capJ*, *capH* (both related to capsule formation), and *coa* (staphylocoagulase enzyme), and the absence of *fnbA, fnbB* (both related to adherence), and *capN* for both isolates belonging to ST25; and the presence of *set21* (exotoxins) in both isolates belonging to ST48. The full complement of genes associated with the type VII secretion system (*esaA, esaB, essA, essB, essC, esxA*) were only found in isolates from ST48 and ST177, which were not clustered together in the phylogenetic analysis.

A total of 677 VF were identified among the 15 isolates in the HIGH SCC IMI category, compared to 670 total VF for the 15 LOW SCC IMI isolates. The median number of VF found in both categories was 44, while the range for the HIGH category was 44-51 and the range for the LOW category was 43-50. There were 61 different VF detected in isolates belonging to the HIGH SCC IMI category, and 57 different VF found in the LOW category. Five VF were unique to the isolates in the HIGH SCC IMI category: *coa, set10, set34, capH* and *capJ.* The two isolates positive for *coa, capH* and *capJ* in the HIGH group were both ST25, which was an ST unique to the HIGH SCC category. The two isolates positive for *set10* and *set34* in the HIGH group belonged to ST136 and ST177, which were both unique to the HIGH SCC IMI category. In the phylogenetic analysis, ST136 and ST177 clustered together 42% of the time, which was below the 65% cutoff used to identify clusters of ST. Only 1 VF was unique to an isolate in the LOW category (*sdrD,* a gene associated with fibrinogen binding proteins rich in aspartic acid and serine). This isolate belonged to ST5 (n=3 isolates), 2 isolates of which were in the HIGH SCC IMI category and did not have the *sdrD* gene, and 1 isolate which was in the LOW category and carried the *sdrD* gene.

In a mixed-effects logistic regression model, total number of VF identified per isolate was not found to be a significant predictor of whether an isolate would be in the HIGH or LOW SCC IMI category (*P* = 0.52). As the number of VF identified was fairly consistent across all isolates in a given ST, statistical analysis exploring if a particular ST (or ST cluster) was a significant predictor of VF number was not possible. All isolates belonging to ST6 (n = 9) and ST25 (n = 2) had 44 VF identified, both isolates in ST48 (n = 2) had 50 VF identified, and 8 of the 9 isolates belonging to ST176 had 44 (1 had 43). The 3 isolates of ST5 had some variation in number of VF (44, 47, 48 genes each).

4.5 Discussion

4.5.1 Diversity of strain type as determined by RAPD and MLST

In all 9 herds, there was at least 1 RAPD type of *S. chromogenes* identified to be causing multiple IMI in quarters belonging to different cows. RAPD-typing has previously been used to compare ST of different isolates of the same species during outbreaks to see if transmission pattern was consistent with infections originating from a common source.In combination with sequencing the 16S rRNA gene for representative isolates, RAPD was used to understand the diversity of ST associated with a multistate outbreak of *Corynebacterium tuberculosis* in several species of animals (Foley et al., 2004), and for investigation of a *Campylobacter jejuni* outbreak in multiple flocks from a single broiler farm (in combination with sequencing the 23S rRNA gene; Payne et al., 1999). RAPD alone was used by Zadoks et al. (2003) to identify transmission dynamics of the mastitis isolate *Streptococcus uberis* within a single herd. In that study, RAPD-typing revealed that each cow was infected with a unique strain. These findings confirmed that the observed mastitis outbreak was not due to contagious transmission, but instead was a result of infections from environmental sources in that herd (Zadoks et al., 2003). Although the objective of the current study was not to identify the transmission dynamics of *S. chromogenes*, identifying the same RAPD type causing IMI in more than one cow in the same herd suggests cow-to-cow spread may be occurring. However, this could also be consistent with transmission via a common point source (Daly et al., 1999). These findings are consistent with Wuytack et al. (2020b) and Reydams et al. (2023), who also used RAPD-typing for *S. chromogenes* isolates and found that a given RAPD type was causing IMI in multiple cows in a herd. Studies using different methods of strain-typing (amplified fragment length polymorphisms: Taponen et al., 2007; PFGE: Gillespie et al., 2009, Mork et al., 2012) have also demonstrated the same *S. chromogenes* strains in IMI from multiple animals in a herd, providing additional evidence that some *S. chromogenes* strains may act in a contagious manner (Jenkins et al., 2019).

Ten different ST (as determined by MLST) were identified for the 30 *S. chromogenes* isolates included in the current study. As the MLST scheme for *S. 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., 2023a and 2023b). In the phylogenetic analysis, study isolates belonging to newly described strain types ST174, ST175, and ST176 were identified as being closely related to ST1 isolates from PubMLST. Furthermore, these 3 ST were identified as single locus variants of ST1 by the MLST 2.0 tool (https://cge.food.dtu.dk/services/MLST/). Isolates in this ST1 cluster were the most frequently found ST in the current study (11/30 isolates, 36.7%). This agrees closely with the work of Huebner et al. (2021), who determined MLST for 120 *S. chromogenes* isolates from Belgium, Vermont (US), and Washington State (US). They found 39/120 (32.5%) of strain-typed isolates belonged to a nodal cluster centered around ST1. For the 48 isolates in Huebner et al. (2021) from Vermont, 36 (75%) belonged to a group they identify as nodal cluster 1. ST1 was also frequently identified in *S. chromogenes* isolates from 2 studies of bovine subclinical IMI in Sweden, which included 132 isolates from 13 herds (Persson Waller et al., 2023a) and 105 isolates from 77 herds (Persson Waller et al., 2023b). However, ST6 and a related novel ST (ST109) were more prevalent in the herds from both Swedish studies. In Huebner et al. (2021), ST1 was the only ST found in all three geographical study locations. ST1 has also been identified in Missouri (US), Canada, and Finland (Fry et al., 2014b; Naushad et al., 2016; https://pubmlst.org/organisms/staphylococcus-chromogenes). ST6 was the second most frequently found ST in the current study (9/30 isolates, 30%), and the third most frequent (15/120, 12.5%) in Huebner et al. (2021). ST6 has also been isolated in Canada and Finland (Naushad et al., 2016; https://pubmlst.org/organisms/staphylococcus-chromogenes). In their two studies of Swedish dairy herds, Persson Waller et al. identified 40 distinct ST among 132 isolates in one study (2023a) and 47 different ST among 105 isolates in another study (2023b). Huebner et al. (2021) found a similar degree of diversity, with 46 ST identified from 120 isolates from 3 geographical locations. After ST1, isolates related to ST15 were the second most commonly identified by Hubener et al. (2021), with 17/120 (14.2%) of isolates belonging to this nodal cluster. Isolates from nodal cluster ST15 were primarily identified in isolates from Vermont and Washington State (16/17 isolates), which Huebner et al. (2021) highlight as an example of geographic variation in the distribution of different ST. Although all isolates in the current study are from Vermont, only 2 of 30 isolates belonged to ST15. Seven and 12 isolates (Persson Waller et al. 2023a, 2023b respectively) related to nodal cluster ST15 described by Huebner et al. (2021) were identified on Swedish dairies. Specifically, in Persson Waller et al. (2023a), 4 isolates belonged to ST15 and 8 to ST19, while in Persson Waller et al. (2023b), 1 isolate belonged to ST15 and 6 to ST19. ST15 has also been identified in Canada (Naushad et al., 2016; https://pubmlst.org/organisms/staphylococcus-chromogenes).

Both Persson Waller et al. (2023b) and Huebner et al. (2021) observed that ST6 and ST1 were both central nodes of ST clusters, with single- and double-locus variants surrounding them. Both authors suggest this indicates a global distribution and subsequent clonal expansion for *S. chromogenes* isolates belonging to these 2 ST. Results of the current study would support this, as the 3 ST in ST cluster 1 (ST174, ST175, ST176) were all newly identified single-locus variants of ST1. Describing the diversity of *S. chromogenes* using MLST is a rapidly growing area of research. Four of the 10 ST in the current study had previously not been described. Forty-five of the 105 isolates (43%) belonged to 33 new ST identified in Persson Waller et al. (2023b), while 33 of the 132 isolates (25%) belonged to 17 new ST identified in Persson Waller et al. (2023a). These results highlight the importance of contributing to public databases to improve our ability to better understand the diversity of this common mastitis pathogen*.*

4.5.2 Associations between ST and SCC category

Our initial hypothesis that ST would be a significant predictor of SCC phenotype (HIGH vs. LOW SCC IMI) was not supported (i.e., we could not reject our null hypothesis). Persson Waller et al. (2023b) also explored associations between genotypes and phenotypic qualities, such as persistency of IMI (over a 1-month period) and association with CMT score at sampling. Although they found no association between ST or ST cluster and persistency, isolates belonging to their cluster VII were significantly more likely to be associated with a high CMT score, indicating a larger inflammatory reaction was occurring in the gland. *S. chromogenes* isolates in Persson Waller et al. (2023b) belonging to ST6 (the most prevalent ST in cluster VII, and the only ST common to our study) tended to be more likely to have a high CMT score vs. other ST in their cluster VII. However, this difference did not achieve the cut-off used for statistical significance (Persson Waller et al., 2023b).

4.5.3 Antimicrobial resistance genes and associations between ARG and SCC category

Overall, both phenotypic resistance and ARG are relatively rare in *S. chromogenes* when compared to other NAS, with the exception of the *blaZ* gene (Sampimon, 2009; Persson Waller et al., 2011). *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).In the current study, *blaZ* was the only ARG identified. Ten of the 30 (33.3%) of isolates were *blaZ-*positive, which is greater than the 10% reported for *S. chromogenes* isolates in a Canadian study (Condas et al., 2017a) and the 22% reported in Persson Waller et al. (2023b), but much less than the 87% of *S. chromogenes* in a Flemish study (Sampimon, 2009). Resistance to β-lactam antibiotics is the predominant type of AMR present in staphylococci, and the reported proportion of NAS isolates exhibiting β-lactamase resistance depends on geographical location (51.6% in Argentina, Raspanti et al. 2016; 63% in South Africa, Phophi et al. 2019; 23% in Belgium and Norway, Fergestad et al. 2021; 14% in Korea, Kim et al. 2019). β-lactam antibiotics are among the few choices for treating mastitis in the US. Within this class, first- and third-generation cephalosporins are the most commonly used, which are more resistant to β-lactamases than penicillin (USDA, 2016; de Campos et al., 2021). In addition to *blaZ*, Persson Waller et al. (2023b) identified *strpS194* (conferring resistance to streptomycin) in 7% of their *S. chromogenes* isolates. This ARG was not found in isolates from the current study. Nobrega et al. (2018) identified various other ARG in *S. chromogenes* isolates, including genes associated with aminoglycoside resistance [*ant(3’’*), *ant(4’), ant(6)*], resistance to amphenicols (*fexA*), and resistance to tetracyclines (*tetK, tetL*). However, the estimated prevalence of these genes in the population of *S. chromogenes* included in their study was low (2-3%).

Carriage of *blaZ* was not found to be a significant predictor of whether an isolate would be associated with a persistently high SCC IMI in the current study. Work exploring the association of ARG carriage and clinical characteristics of IMI in *S. chromogenes* is limited, but previous research identified a link between phenotypic resistance in *S. aureus* and clinical IMI outcome. Both Sol et al. (2000) and Taponen et al. (2003) report that penicillin-resistant strains of *S. aureus* (those which produced β-lactamase) had a lower bacteriological cure rate *in vivo,* despite use of an appropriate intramammary antibiotic that the isolate was susceptible to *in vitro*. *S. aureus* isolates in Sol et al. (2000) were associated with clinical mastitis, whereas isolates in Taponen et al. (2003) were from cases of subclinical mastitis. Further, Sol et al. (2000) reported that IMI due to penicillin-resistant *S. aureus* were associated with a more persistently elevated SCC, indicating the IMI was associated with a higher degree of inflammation. Both Sol et al. (2000) and Taponen et al. (2003) conclude that either: 1) the penicillin-resistant strains of *S. aureus* were more refractory to treatment than susceptible strains due to a possible relationship between production of β-lactamase and some other unidentified virulence factors, or 2) that any antibiotic used to treat mastitis caused by penicillin-resistant strains works less efficiently, due to unidentified pharmacokinetic or pharmacodynamic factors. A more recent example of an association between ARG and clinical characteristics of an IMI due to NAS is described in Wuytack et al. (2020a). When comparing NAS isolates associated with IMI which had an SCC of ≤50,000 cells/mL to isolates from cases of clinical mastitis, Wuytack et al. (2020a) identified *mecA* (a methicillin-resistance gene) in 21/43 (49%) of NAS isolates originating from clinical mastitis and only 1/16 (6%) isolates from quarters with an SCC of ≤50,000 cells/mL. Based on these findings, the authors suggest that *mecA* in NAS isolates from bovine IMI may be linked to virulence genes or pathogenicity islands, supposedly both present on a mobile genetic element (*SCCmec,* staphylococcal cassette chromosome *mec).* Of the 22 NAS isolates identified as *mec*-positive in Wuytack et al. (2020a), none were *S. chromogenes.* Further research exploring associations between ARG and clinical characteristics of IMI including a larger number of *S. chromogenes* is certainly warranted, to better understand if particular undesirable traits (e.g., penicillin resistance and a greater inflammatory response) are genetically linked in this ubiquitous mastitis pathogen.

Although we did not find any support for an association between carriage of *blaZ* and the associated SCC category of an IMI, results from the current study suggest that *blaZ* carriage is likely a function of ST in *S. chromogenes.* For all but 1 of the 5 MLST identified, *blaZ* carriage was uniform across a ST. Numerous studies have identified that resistance profiles for NAS are species-specific (Sampimon, 2009; Persson Waller et al., 2011; Taponen et al., 2016; Nobrega et al., 2018; Fergestad et al., 2021; Taponen et al., 2023), so a genetic basis for carriage of particular AMR determinants at the strain level would not be surprising. For *S. aureus*, carriage of methicillin resistance has been associated with particular clonal complexes both in human medicine (Smith et al., 2021; Garrine et al., 2023) and certain clusters of *spa* ­type for bovine clinical mastitis isolates (Freu et al., 2022). Additionally, in a study comparing isolates from persistent and nonpersistent *S. aureus* IMI*,* Haveri et al. (2007) found that a particular pulsotype associated more with persistent IMI was significantly more likely to harbor the *blaZ* gene. An association between genetic grouping and *blaZ* carriage in *S. chromogenes* was identified in Persson Waller et. al (2023b). In their study, all isolates of ST19, ST102, and ST103 carried *blaZ*. When analyzing clusters of ST, the two clusters comprised primarily of these 3 ST (clusters III and IV) were more likely to be *blaZ*-positive than other clusters of ST. As isolates belonging to these ST were distributed over different farms and counties in Sweden, the authors suggest that *blaZ-*mediated penicillin resistance is likely a result of the spread of certain lineages of *S. chromogenes,* instead of horizontal gene transfer between different strains or species (Persson Waller et al., 2023b). Three of the 4 ST which had uniform *blaZ* carriage in the current study were also distributed over multiple farms. Consistent carriage of *blaZ* from ST originating from different farms may suggest that *blaZ* is located chromosomally for these *S. chromogenes* isolates, instead of on a plasmid. Location of *blaZ* carriage is not well characterized for *S. 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). They also characterized the diversity of *blaZ* genes among the *S. aureus* isolates, identifying 6 different protein signatures. Studies exploring whether *blaZ* is more likely to be carried chromosomally or on a plasmid for *S. chromogenes* from bovine IMI, as well as characterizing the genetic diversity of the gene present in this population of isolates, would be useful in understanding potential mechanisms of transmission of penicillin resistance for this predominant mastitis pathogen.

4.5.4 Virulence genes and associations between VF and SCC category

The overall number of unique VF identified in the current study (62) from 30 *S. chromogenes* isolates was similar to the findings of Persson Waller et al. (2023b), who identified 57 unique genes among the 105 *S. chromogenes* isolates from Sweden. The mean number of VF per isolate reported by Persson Waller et al. (2023b) was 30 (SD: 5.4, range: 25-45), which is somewhat lower than the median (44) and range (43-51) reported for isolates in the current study. The database and methodology for identifying VF used in the current study is consistent with Persson Waller et al. (2023b), facilitating a direct comparison of these values. In a smaller-scale study of 8 *S. chromogenes* from Finland using a different database, a range of 37-49 VF were identified (Åvall-Jääskeläinen et al., 2018). Although separate species-specific summary statistics are not provided for the 83 *S. chromogenes*, Naushad et al. (2019) found a mean of 30 VF genes each for the 441 NAS isolates from 25 different species. They report that the phylogenetic grouping of NAS species with the highest virulence potential (defined by total number of VF) was clade B, which contains *S. chromogenes, S. agnetis,* and *S. hyicus*. A proportionately higher number of exotoxin genes, host evasion genes, and capsular genes contributed to clade B’s high virulence potential in their study.

Biofilm formation has been proposed as an important virulence factor by which staphylococci causing intramammary infections evade the cow's immune response, leading to persistent intramammary infections (for NAS: Simojoki et al., 2012 Tremblay et al., 2013; for *S. aureus*: Keefe, 2012). Biofilms consist of a structured surface-associated community of bacterial cells, and are characterized by an extracellular polysaccharide matrix (Tolker-Nielsen, 2015). Biofilms shield bacteria from the host’s immune defenses by inhibiting phagocytosis and neutralizing antimicrobial peptides (Costerton et al., 1999), while also potentially enhancing resistance to antimicrobial treatments (Fey and Olson, 2010). While multiple factors contribute to biofilm formation in staphylococci, it is closely associated with production of the surface protein Bap (biofilm-associated protein), encoded by the *bap* gene (Otto, 2013). *bap* has been shown to confer a strong biofilm-forming phenotype in *S. aureus* mastitis isolates (Lasa and Penadés, 2006). However, a study including various staphylococcal species found no significant association between the presence of *bap* and biofilm production (Francisco et al., 2021). *bap* was not detected in any of the 83 *S. chromogenes* isolates in Naushad et al. (2019), any of the 22 *S. chromogenes* isolates in Simojoki et al. (2012), or any of the 25 isolates of *S. chromogenes* included in a Belgian study of clinical and low-SCC IMI (Wuytack et al., 2020a). It was also rare in Åvall-Jääskeläinen et al. (2018), where it was only identified in 1/8 *S. chromogenes* isolates, and was somewhat sporadically found in Persson Waller et al. (2023b) in 13/105 isolates. In the 255 coagulase-negative staphylococci isolates included in Tremblay et al. (2013), only 37 isolates (14.5%) were *bap* positive. They note that the distribution of *bap* among species varied considerably: *bap* was rare in *S. chromogenes* and *S. simulans*, but was detected in almost every *S. xylosus* isolate (92%; Tremblay et al., 2013). In light of these findings, it is notable that *bap* was identified in 28 of the 30 *S.* chromogenes isolates (93.3%) in the current study. *bap*-positive *S. aureus* may persist longer in the mammary gland and exhibit increased antimicrobial resistance through biofilm formation (Cucarella et al., 2002, 2004), though other studies found no significant difference in antimicrobial susceptibility between biofilm and non-biofilm-producing *S. aureus* (Fidelis et al., 2024). Zuniga et al. (2015) report that staphylococcal isolates (NAS and *S. aureus*) positive for a group of adhesin genes (*eno, fnbA,* and *fib*) and *bap* originated from quarters with higher SCC when compared to isolates from quarters which were negative for these genes, as well as compared to culture-negative quarters. The authors propose that the presence of these genes may be related to an increased inflammatory response (Zuniga et al., 2015). For NAS specifically, it has been suggested that biofilms increase their ability to persist in the mammary environment (Tremblay et al., 2013; Piessens et al, 2012; Zuniga et al., 2015). As all 30 isolates in the current study are from persistent IMI, finding *bap* in such a high proportion is consistent with the notion that biofilms play a role in the ability of *S. chromogenes* to cause chronic infections. However, some work indicates that biofilm formation by NAS does not appear to contribute to infection persistence or provoke a stronger inflammatory response (Simojoki et al., 2012). As the relationship between pathogenicity and biofilm formation is not clear for NAS causing bovine intramammary infections, additional *in vivo* testing is needed (De Buck et al., 2021). As reviewed by Pederson et al. (2021), *in vitro* observations of biofilm formation are not easily comparable with what happens *in vivo* within the bovine mammary gland.

Another staphylococcal virulence factor proposed to play a role in evasion of the host immune response is a polysaccharide capsule which resists phagocytic cell uptake. In Naushad et al. (2019), genes encoding capsular proteins A-L and N were identified in less than 15% of isolates, while *capM,* *capO,* and *capP* genes were identified in 100, 100, and 96% of isolates respectively. Only 1 of 8 *S. chromogenes* in Åvall-Jääskeläinen et al. (2018) had any capsular genes, and only 2/8 isolates associated with clinical mastitis in Wuytack et al. (2020a) were positive for *cap5H* by PCR. All 30 isolates in the current study contained at least 3 different *cap* genes, with 28 having *capN, capO,* and *capP.* Two isolates in the current study were missing *capN,* but possessed both *capJ* and *capH.* There is conflicting evidence on the associations between capsule genes and overall virulence of staphylococci causing bovine IMI. Some evidence exists that staphylococci lacking a capsule can better persist in the mammary gland, as encapsulated strains elicit more inflammation and are thereby eliminated faster (Tuchscherr et al., 2005). Other research suggests that the antiphagocytic properties of the capsule allows staphylococci to persist in infected hosts (Thakker et al., 1998). Citing work showing that lack of a capsule is advantageous for *S. aureus* causing chronic IMI, Naushad et al. (2019) argue that the low prevalence of capsule genes in their *S. chromogenes* isolates may explain the how the pathogen has become so widespread in the population of Canadian dairy animals. Finding such a large proportion of isolates carrying multiple capsule genes in the current study of *S. chromogenes* isolates from persistent IMI instead supports the idea that a capsule enhances the ability of the organism to evade the host’s immune response. More research is needed to test this hypothesis.

Staphylococcal complement inhibitor (encoded by the gene *scn*) also plays a role in the ability of staphylococci to evade the host’s immune system. *scn* encodes a protein which inhibits the complement system, reducing phagocytosis of the bacterium following opsonization. Although staphylococcal complement inhibitor had been thought to be highly specific to isolates of human origin, Naushad et al. (2019) detected *scn* in a high proportion (88%) of *S. chromogenes* isolates in their study. In agreement with these findings, all 30 *S. chromogenes* isolates in the current study were positive for *scn.* Adenosine synthase A (*adsA*) is an immune evasion factor identified in *S. aureus,* which is responsible for increasing the amount of extracellular adenosine, a potent immuno-suppressive signaling molecule. *adsA* allows staphylococci to survive after being engulfed by neutrophils, giving it the ability to evade the bactericidal activity of host leukocytes. *adsA* was found in a high proportion (99%) of isolates from Naushad et al. (2019), and all 30 isolates in the current study.

Another widely found VF in *S. chromogenes* is β-hemolysin, a phospholipase C toxin secreted by *S. aureus*. β-hemolysin was the most frequently found gene in *S. chromogenes* isolates and other species of clade B in Naushad et al. (2019), was found in all 8 isolates in (Åvall-Jääskeläinen et al., 2018), and all 30 of the isolates in the current study. In contrast, the gene encoding exfoliative toxin type C (*etc,* which causes the loss of cell‐cell adhesion in the superficial epidermis in humans) was not identified in any of the *S. chromogenes* isolates in Naushad et al. (2019) or (Åvall-Jääskeläinen et al., 2018), but was present in all 30 of the isolates in the current study. Exfoliative toxins in NAS have been identified in *S. agnetis* and *S. chromogenes* from broiler chickens (as reviewed in Szafraniec et al., 2020), but are not widely reported from isolates of bovine IMI. Another set of toxin genes commonly identified in NAS is the β-type phenol-soluble modulins (PSMs), which have been shown in *S. aureus* to cause lysis of red and white blood cells, contributing to biofilm development and stimulation of inflammatory responses in the host. In Naushad et al. (2019), all *S. chromogenes* isolates possessed a single gene associated with PSMs (*PSMβ4*)*,* which was also widely found in isolates from Persson Waller et al. (2023b). All isolates in the current study had the entire suite of PSM-associated genes described in the comprehensive NAS database (*PSMβ1- PSMβ6*), although more research is needed to understand the significance of having a larger number of genetic determinants associated with PSMs for the pathogenesis of *S. chromogenes.*

Two *S. chromogenes* isolates in the current study were positive for *coa,* the gene encoding for the staphylocoagulase enzyme. Staphylocoagulase binds to prothrombin in the host, ultimately forming a fibrin clot which shields the bacteria from the host's defenses and causes localized clotting. *coa* has previously been identified in *S. agnetis* and *S. hyicus* from bovine IMI*,* which are considered coagulase variable (Vanderhaeghen et al., 2015). Except for *S. aureus, S. hyicus,* and *S. agnetis*, coagulase positive staphylococci are rarely isolated from bovine IMI, which is why the coagulase test has been so widely used to classify staphylococci from mastitis into coagulase-positive (primarily *S. aureus*) and coagulase-negative (largely, all other species of staphylococci) (Vanderhaeghen et al., 2015). None of the 441 NAS isolates in Naushad et al. (2019) were *coa-*positive, while 4/4 *S. agnetis* but 0/8 *S. chromogenes* were *coa-*positive in Åvall-Jääskeläinen et al. (2018). Carriage of the *coa* gene by *S. chromogenes* from bovine IMI has not yet been widely reported. Another protein exhibiting coagulating ability, the von Willebrand factor-binding protein, is widely present in NAS bovine IMI isolates. All 30 isolates in the current study were positive for *vWbp,* as were 94% of *S. chromogenes* isolates in Naushad et al. (2019).

In the current study, our hypothesis that total number of virulence genes for each isolate would be a significant predictor of whether it was associated with a HIGH or LOW SCC IMI was not supported. Other researchers exploring virulence potential in NAS of bovine origin have come to similar conclusions. In their study of VF found in 4 different staphylococcal species (4 isolates each of *S. aureus* and *S. agnetis,* 8 isolates each of *S. chromogenes,* and *S. simulans*), Åvall-Jääskeläinen et al. (2018) found no association by visual inspection between the type of mastitis (clinical vs. subclinical) and specific virulence genes, virulence gene profiles, or the cumulative number of virulence genes. As statistical power to analyze these relationships by species in their study was limited by number of isolates, logistic regression analyses of pooled data for all NAS isolates was carried out; still, they did not observe any clear difference in the virulence gene profiles or cumulative number of virulence genes between isolates from clinical and subclinical mastitis (Åvall-Jääskeläinen et al., 2018). Most of the isolates had unique virulence gene profiles, and when two isolates of the same species shared an identical profile, 1 of the isolates was clinical while the other was subclinical (Åvall-Jääskeläinen et al., 2018). When comparing isolates from clinical and subclinical mastitis caused by *S. aureus,* Haveri et al. (2007) found no difference in the cumulative number of VF between the two groups. In a Canadian study of 255 NAS IMI isolates, no association between biofilm formation and SCC associated with the IMI was observed (Tremblay et al., 2013). Similarly, no association was found between the phenotypic ability of a NAS isolate to form biofilm and the persistence of IMI when isolates from 63 persistent and 55 transient IMI were compared (Simojoki et al., 2012). In the same study, researchers found no association between the ability of 114 NAS isolates to form biofilms and the severity of the associated mastitis (as measured by milk N-acetyl-b-D-glucosaminidase activity, an enzyme which reflects tissue damage and is an indicator of inflammation in the udder; Simojoki et al., 2012).

In contrast, other researchers have identified associations between clinical characteristics of an IMI and VF of NAS isolates causing the infections. 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 VF for a NAS isolate was associated with an increase in the logSCC of the associated IMI. However, when stratified by type of virulence, the presence of each additional toxin gene for a NAS 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 VF was not associated with increased severity of an IMI (Naushad et al., 2019). In a regression analysis with VF stratified by functional category, the presence of each additional VF gene associated with host immune evasion increased the odds of having a more severe immune response by 0.074 (Naushad et al., 2019). Naushad et al. (2019) applied various approaches in order to determine whether particular VF distributions had any association with SCC category or occurrence of clinical mastitis, but no clustering of isolates representing low SCC, medium SCC, or high SCC or clinical mastitis was identified. For *S. chromogenes* specifically, Persson Waller et al. (2023b) also identified various associations between clinical characteristics of an IMI and VF. They found that a higher number of exoenzyme genes were present in isolates associated with milk samples that had a low CMT vs. a high CMT (Persson Waller et al., 2023b). Additionally, isolates from low CMT quarters had higher number of evasion genes than those with high CMT, and the *geh* gene (encoding a lipase) specifically was associated with increased odds of having a low CMT (Persson Waller et al., 2023b). As these findings contrasted with those of Naushad et al. (2019) described above, Persson Waller et al. (2023b) were unable to identify why they may have observed this association. In this study, all 30 isolates from both HIGH and LOW SCC categories were positive for *geh.* This contrasts with Persson Waller et al. (2023b), where *geh* was found predominantly in isolates from IMI cases associated with lower levels of inflammation. Persson Waller et al. (2023b) also found that presence of the *capJ* and *capH* genes were significantly associated with IMI that came from quarters with a lower CMT. This would be consistent with encapsulated staphylococci being better able to persist in the udder, as they may cause less of an inflammatory response (Thakker et al., 1998). In contrast, the only 2 isolates positive for *capJ* and *capH* in the current study were associated with a persistently high SCC IMI.

As evidenced by the results of the current study and others failing to find a link between the cumulative number of VF found in staphylococci from a bovine IMI and the degree of inflammation associated with the infection, the expression of disease in an individual animal and the interactions of various VF with the host’s immune system are complex. Åvall-Jääskeläinen et al. (2018) suggest it is likely that similar symptoms can be caused by several different combinations of virulence factors, rather than by any particular one alone. Similarly, the progression of disease may be determined by the interplay of various VF rather than just the presence of any specific virulence gene. Evidence in support of this was found in Naushad et al. (2019), where they analyzed the relationships between the patterns of VF associated with isolates from low, medium, and high SCC and clinical mastitis. They were able to demonstrate unique patterns of associations between VF for low SCC and CM isolates, with many distinct positive and negative association patterns for clinical mastitis isolates in particular. In regards to NAS and IMI, De Buck et al. (2021) write that “pathogenesis is complex and often involves an organized and systematic participation of various VFs to establish disease,” and that “often VFs complement each other to promote pathogen colonization and persistence of disease.” The impact of virulence genes on disease outcomes or development is likely affected by intrinsic (host-level) factors, including the host’s environment, nutritional status, and genetics. A particular example of this is the increased concentration of lactoferrin in mastitic milk, which likely inhibits the ability of staphylococci to form biofilms in the udder (as summarized in Simojoki et al., 2012). Extrinsic (environmental) factors, including herd management practices, climatic conditions, the presence of other pathogens in the environment, also play important roles in the successful colonization, persistence, and virulence capability of staphylococci causing intramammary infections.

Similar to *blaZ*, the carriage of VF by isolates in the current study appears to be more a function of phylogeny than a predictor of infection phenotype (as measured by qSCC). The cumulative number of VF identified belonging to the 5 ST with multiple isolates showed little to no variation. Total number of VF identified per isolate was uniform for 3 of these 5 ST (ST25, ST6, and ST48), 8 of 9 isolates belonging to ST176 contained the same number of VF (with the remaining isolate differing by 1), and isolates belonging to ST5 ranged from 44-48 VF identified per isolate. In a visual assessment of the heat map of VF with isolates organized by ST (Figure 4.3), many of the limited differences in presence or absence of VF occurred at the ST level. The only isolates lacking *fnbA,* *fnbB*, and *capN*, and possessing *coa, capH* and *capJ* both belong to ST25. The only isolates possessing the staphylococcal exotoxin gene *set21* were the 2 isolates in ST48. Two isolates of singleton ST (ST177 and ST136) which were not classified as a cluster but grouped together with a bootstrap value of 42% of the time in the phylogenetic analysis were the only 2 isolates positive for the staphylococcal exotoxin genes *set10* and *set34.* Support for an association between phylogeny and VF presence in *S. chromogenes* was also found in Persson Waller et al. (2023b). When analyzed at the level of ST cluster, isolates belonging to cluster III, IV, and VII had fewer VF compared to isolates belonging to other clusters, and cluster IV had significantly more exoenzyme genes vs. other clusters. At the strain level, they identified ST59 had higher number of adherence genes vs. other ST. The only gene identified to be associated with phylogenetic grouping was *atl* (autolysin), which was present in clusters V, VI, and VII but absent in II, III, and IV (Persson Waller et al., 2023b). In the current study, *atl* was consistently found in all 30 *S. chromogenes* isolates. 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, NAS isolates instead grouped together by their respective species. As the pattern of virulence genes carried by NAS isolates likely is species-dependent, a genetic basis for carriage of VF may also extend to the strain level, although more research is needed to support this finding.

A limitation to the current study is that only isolates from persistent IMI are included. This study design was selected to explore the effect of pathogen factors on qSCC while controlling for other variables (effect of cow and farm). Future studies could include isolates associated with transient IMI, as well as isolates from other locations on the cow (specifically, those present on the teat apex or colonizing the streak canal). As isolates in the current study were collected using the conventional milk sampling technique, it is difficult to distinguish if they originate from teat apex or streak canal colonization or from IMI (Hiitiö et al., 2016).

Our ability to perform a formal statistical analysis in order to explore if pattern and number of VF vary by ST was limited both by the relatively small number of isolates assigned to most ST identified in the current study, as well as complete uniformity in the number and type of VF carried by a particular ST. The number of *S. chromogenes* (30) in this study which were submitted for WGS was a function of financial constraint. Future studies with larger isolate collections, isolates collected from a greater number of farms, and the ability to sequence a larger number of *S. chromogenes* isolates may be better able to explore associations of ARG and VF 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. An additional limitation in the methodology of this and related studies on VF in NAS of bovine origin (Persson Waller et al., 2023b; Naushad et al., 2019) is that the database used to identify VF and predict their function was extrapolated from *S. aureus* causing bovine IMI or NAS which were isolated from humans. Until research elucidating the specific pathogenesis for VF identified in NAS isolates of bovine origin is carried out, we are left to infer that VF which are genetically similar to those that are better described in other populations of staphylococci are relevant in bovine IMI. The database compiled and distributed by Naushad et al. (2019) and used in the current study is an extremely valuable contribution to our field, and provides a solid and extensive foundation from which to extend our understanding of VF present in NAS causing IMI in dairy cattle. Lastly, the simple presence or absence of a virulence gene is not indicative of how it is expressed by a pathogen causing an infection in the udder. Previous work on *S. aureus* has shown that the expression level of the VF may influence disease outcome in mastitis (Le Maréchal et al., 2011). Studies exploring gene expression by *S. chromogenes* while causing an IMI would elucidate its pathogenicity *in vivo*.

4.6 Conclusions

For isolates included in the current study, ST (as determined by MLST) of *S. chromogenes* was not associated with persistently HIGH or LOW SCC IMI. Ten different ST were identified among the 30 isolates, including 4 novel ST. Seven ST were identified in each SCC category, with 4 ST found in both, 3 unique to HIGH, and 3 unique to LOW. The most common ST were ST6 and ST176, with 18 isolates (60%) belonging to 1 of these 2 ST. The only ARG identified was *blaZ,* encoding for resistance to penicillin (33.3% of isolates). Sixty-two unique VF were detected, with a median of 44 VF per isolate, and a range of 43-21. Neither overall number of VF nor *blaZ* carriage was found to be a significant predictor of SCC category. *blaZ* carriage, number, and type of VF appears to be a function of ST for *S. chromogenes*, but more research is needed to confirm these findings.

4.7 Notes

This project was funded by the Organic Agriculture Research and Extension Initiative (OREI) from the National Institute of Food and Agriculture (USDA-NIFA grant 2018-51300-28561). Work on this project was also supported by Vermont Experiment Station USDA Animal Health and Disease Research Projects VT-AH02703 and VT-2023-AH01, and subaward C00075322-1 under USDA-NIFA project 2022-67015-37123 with Dr. Adkins. Dr. Barlow also contributed effort to this project during a sabbatical year with partial salary support from USDA-NIFA project 2023-69015-40254. The first author, Caitlin E. Jeffrey was supported by a USDA-NIFA Predoctoral Fellowship award (grant 2022-67011-36567). Supplemental data is available at https://zenodo.org/records/13255274. The authors thank the organic dairy producers who agreed to participate in this study, for giving us their time and allowing us to collect samples from their farms. We are grateful to the numerous University of Vermont undergraduate students who assisted with sample collection. We thank the laboratory staff at the Vermont State Agricultural and Environment Laboratory for determination of somatic cell counts from the quarter milk samples. We thank Paige Isensee, Natalie Sexton, Madyson Marrs, and Allena Radford, who provided laboratory assistance in the Adkins lab at the University of Missouri. Caitlin Jeffrey conceptualized the study, acquired funding, coordinated farm recruitment and sampling, conducted on-farm sample collection, completed work associated with strain-typing (RAPD and MLST), managed and curated the data, conducted the data analysis, prepared data visualizations and presentation, and wrote the original draft. Ashma Chakrawarti identified antimicrobial resistance genes and virulence factors from assembled genomes. Pamela Adkins conducted isolate species identification by MALDI-TOF, completed comparative analysis of RAPD types using BioNumerics Software, and reviewed and edited the manuscript. John Barlow conceptualized the study, acquired funding, supervised the research, conducted on-farm sample collection, and reviewed and edited the manuscript. The authors have not stated any conflicts of interest.

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4.9 Tables

|  |  |  |
| --- | --- | --- |
| Table 4.1 Diversity of RAPD types causing persistently high or low somatic cell count (SCC) *Staphylococcus chromogenes* intramammary infections (IMI) by farm. An *S. chromogenes* IMI was considered persistent if it had ≥ 2 quarter-day observations (from sequential sampling events approximately 30 days apart) and was infected with *S. chromogenes* only for all quarter-day observations. Persistent IMI were then selected where all quarter-day observations had an associated SCC of ≥200,000 cells/mL or <200,000 cells/mL. All isolates associated with each IMI were strain-typed using RAPD to ensure persistency; 74 out of 75 IMI were found to be caused by the same strain of *S. chromogenes.* | | |
| Farm | No. persistent IMI | No. RAPD types |
| Farm A | 8 | 3 |
| Farm C | 14 | 9 |
| Farm D | 6 | 3 |
| Farm E | 7 | 5 |
| Farm F | 3 | 2 |
| Farm G | 8 | 5 |
| Farm H | 13 | 7 |
| Farm I | 3 | 2 |
| Farm J | 11 | 6 |

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| --- | --- | --- | --- | --- | --- | --- |
| Table 4.2 Farm of origin, cow, somatic cell count (SCC) category, parity, and average days in milk (DIM) at time of sampling associated with 30 persistent *Staphylococcus chromogenes* intramammary infections (IMI). Avg. DIM is the mean DIM of the cow over repeated sampling events (n=2 or 3). An *S. chromogenes* IMI was considered persistent if it had ≥ 2 quarter-day observations (from sequential sampling events approximately 30 days apart) and was infected with *S. chromogenes* only for all quarter-day observations. All isolates in the HIGH category come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations. TS = tiestall; BP = bedded pack farm. | | | | | | |
| IMI | Farm | Cow | SCC category | Parity | Avg. DIM | Range of SCC for IMI (cells/mL) |
| 1 | BP-1 | A | high | 2 | 255 | 220,000 - 240,000 |
| 2 |  | B | low | 1 | 240 | 83,000 - 99,000 |
| 3 |  | C | low | 3 | 217 | 120,000 - 140,000 |
| 4 | BP-2 | A | high | 6 | 91 | 210,000 - 530,000 |
| 5 | TS-2 | A | high | 1 | 438 | 200,000 - 390,000 |
| 6 |  | A | low | 1 | 438 | 67,000 - 130,000 |
| 7 |  | B | high | 4 | 119 | 200,000 - 620,000 |
| 8 |  | C | low | 6 | 107 | 67,000 - 72,000 |
| 9 | BP-3 | A | low | 4 | 41 | 94,000 - 180,000 |
| 10 |  | B | high | 4 | 58 | 200,000 - 350,000 |
| 11 |  | C | low | 4 | 231 | 180,000 - 190,000 |
| 12 |  | D | low | 2 | 155 | 52,000 - 120,000 |
| 13 |  | E | low | 2 | 229 | 40,000 - 170,000 |
| 14 | TS-3 | A | high | 3 | 293 | 300,000 - 580,000 |
| 15 |  | B | low | 1 | 288 | 8,000 - 43,000 |
| 16 |  | B | low | 1 | 288 | 13,000 - 130,000 |
| 17 |  | C | low | 3 | 150 | 29,000 - 110,000 |
| 18 |  | D | high | 3 | 293 | 410,000 - 1,000,000 |
| 19 |  | E | low | 1 | 43 | 20,000 - 61,000 |
| 20 |  | F | high | 3 | 307 | 230,000 - 1,400,000 |
| 21 |  | G | low | 3 | 323 | 110,000 - 190,000 |
| 22 |  | H | high | 1 | 318 | 240,000 - 580,000 |
| 23 |  | H | high | 1 | 318 | 770,000 - 1,400,000 |
| 24 | BP-4 | A | low | 2 | 274 | 137,000 - 170,000 |
| 25 |  | B | high | 2 | 277 | 616,000 - 770,000 |
| 26 |  | C | high | 1 | 281 | 310,000 - 690,000 |
| 27 |  | C | high | 1 | 281 | 2,496,000 - 3,100,000 |
| 28 |  | C | high | 1 | 281 | 349,000 - 370,000 |
| 29 |  | D | high | 4 | 196 | 233,000 - 340,000 |
| 30 | BP-5 | A | low | 4 | 86 | 57,000 - 140,000 |

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| Table 4.3 Number of *Staphylococcus chromogenes* isolates associatedwith persistent bovine intramammary infections (IMI) belonging to each strain type [ST; as determined by multilocus sequence typing (MLST)], stratified by somatic cell count (SCC) category. All isolates in the HIGH category come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations. A phylogenetic tree was constructed from concatenated nucleotide sequence data for the study isolates as well as 386 publicly available concatenated MLST sequences for *S. chromogenes.* Study isolates which grouped together with a bootstrap value of ≥65% were classified as ST clusters. | | | |
| SCC category | Strain type | ST cluster | No. isolates |
| HIGH | 5 | 5 | 1 |
|  | 6 | 6 | 5 |
|  | 25 | 25 | 2 |
|  | 48 | 48 | 1 |
|  | 136 | - | 1 |
|  | 176\* | 1 | 4 |
|  | 177\* | - | 1 |
| LOW | 5 | 5 | 2 |
|  | 6 | 6 | 4 |
|  | 48 | 48 | 1 |
|  | 51 | - | 1 |
|  | 174\* | 1 | 1 |
|  | 175\* | 1 | 1 |
|  | 176\* | 1 | 5 |
| \* Indicates a novel strain type of *S. chromogenes* not previously identified in PubMLST | | | |

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| Table 4.4 *blaZ* gene carriage by strain type (as determined by multilocus sequence typing) and ST cluster for 30 *Staphylococcus chromogenes* isolates associatedwith persistent bovine intramammary infections. Study isolates which grouped together with a bootstrap value of ≥65% in a phylogenetic analysis were classified as ST clusters. | | |
| Strain type | ST cluster | Prop. isolates + for *blaZ* (%) |
| 6 | 6 | 4/9 (44%) |
| 176 | 1 | 0/9 (0%) |
| 5 | 5 | 3/3 (100%) |
| 25 | 25 | 0/2 (0%) |
| 48 | 48 | 2/2 (100%) |
| 51 | - | 1/1 (100%) |
| 136 | - | 0/1 (0%) |
| 174 | 1 | 0/1 (0%) |
| 175 | 1 | 0/1 (0%) |
| 177 | - | 0/1 (0%) |

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| --- | --- | --- | --- | --- |
| Table 4.5 Presence of potential virulence factors and related genes for 30 *Staphylococcus chromogenes* isolates from subclinical bovine intramammary infections (IMI), stratified by somatic cell count (SCC) category. All isolates in the HIGH category (n = 15) come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW (n = 15) category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations. Grouping scheme adapted from Naushad et al., 2019. Bolded virulence factors with an asterisk represent related genes that were unique to an SCC category. | | | | |
| Type of virulence |  |  | SCC category | |
| *Adherence* | Virulence factor | Related genes | LOW  n (%) | HIGH  n (%) |
|  | Autolysin | *atl* | 15 (100) | 15 (100) |
|  | Biofilm-associated surface protein | *bap* | 14 (93.3) | 13 (86.7) |
|  | Fibronectin binding proteins | *fnbA* | 15 (100) | 13 (86.7) |
|  | Fibronectin binding proteins | *fnbB* | 15 (100) | 13 (86.7) |
|  | Ser-Asp-rich fibrinogen binding proteins | *sdrC* | 1 (6.7) | 1 (6.7) |
|  | **Ser-Asp-rich fibrinogen binding proteins\*** | *sdrD* | 1 (6.7) | 0 (0) |
|  | Ser-Asp-rich fibrinogen binding proteins | *sdrE* | 1 (6.7) | 1 (6.7) |
|  | Ser-Asp-rich fibrinogen binding proteins | *sdrG* | 1 (6.7) | 1 (6.7) |
| *Host immune evasion* |  |  |  |  |
|  | **Capsule formation\*** | *capH* | 0 (0) | 2 (13.3) |
|  | **Capsule formation\*** | *capJ* | 0 (0) | 2 (13.3) |
|  | Capsule formation | *capN* | 15 (100) | 13 (86.7) |
|  | Capsule formation | *capO* | 15 (100) | 15 (100) |
|  | Capsule formation | *capP* | 15 (100) | 15 (100) |
|  | Staphylococcal complement inhibitor | *scn* | 15 (100) | 15 (100) |
| *Iron uptake and metabolism* |  |  |  |  |
|  | ABC transporter (siderophore receptor) | *htsA* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *htsB* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *htsC* | 15 (100) | 15 (100) |
|  | Iron-regulated surface determinant protein | *isdF* | 15 (100) | 15 (100) |
|  | Iron-regulated surface determinant protein | *isdG* | 15 (100) | 15 (100) |
|  | Iron-regulated surface determinant protein | *isdI* | 15 (100) | 15 (100) |
|  | Staphyloferrin B synthesis-related genes | *sbnA* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaA* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaB* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaC* | 15 (100) | 15 (100) |
|  | ABC transporter (siderophore receptor) | *sfaD* | 15 (100) | 15 (100) |
|  | Staphyloferrin A synthesis-related | *sirA* | 15 (100) | 15 (100) |
|  | Staphyloferrin A synthesis-related | *sirB* | 15 (100) | 15 (100) |
|  | Staphyloferrin A synthesis-related | *sirC* | 15 (100) | 15 (100) |
| *Exoenzymes* |  |  |  |  |
|  | Adenosine synthase A | *adsA* | 15 (100) | 15 (100) |
|  | Aureolysin | *aur* | 15 (100) | 15 (100) |
|  | **Staphylocoagulase\*** | *coa* | 0 (0) | 2 (13.3) |
|  | Lipase | *geh* | 15 (100) | 15 (100) |
|  | Lipase | *lip* | 15 (100) | 15 (100) |
|  | Thermonuclease | *nuc* | 15 (100) | 15 (100) |
|  | von Willebrand factor-binding protein | *vWbp* | 15 (100) | 15 (100) |
| *Exotoxins* |  |  |  |  |
|  | **Staphylococcal exotoxin 10\*** | *set10* | 0 (0) | 2 (13.3) |
|  | Staphylococcal exotoxin 15 | *set15* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 16 | *set16* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 18 | *set18* | 1 (6.7) | 2 (13.3) |
|  | Staphylococcal exotoxin 20 | *set20* | 13 (86.7) | 10 (66.7) |
|  | Staphylococcal exotoxin 21 | *set21* | 1 (6.7) | 1 (6.7) |
|  | Staphylococcal exotoxin 26 | *set26* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 3 | *set3* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 30 | *set30* | 15 (100) | 15 (100) |
|  | **Staphylococcal exotoxin 34\*** | *set34* | 0 (0) | 2 (13.3) |
|  | Staphylococcal exotoxin 40 | *set40* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 6 | *set6* | 15 (100) | 15 (100) |
|  | Staphylococcal exotoxin 8 | *set8* | 1 (6.7) | 2 (13.3) |
| *Toxins: Phenol soluble modulins* |  |  |  |  |
|  | Phenol soluble modulins (β) | *PSMB1* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB2* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB3* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB4* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB5* | 15 (100) | 15 (100) |
|  | Phenol soluble modulins (β) | *PSMB6* | 15 (100) | 15 (100) |
| *Toxins: Hemolysins* |  |  |  |  |
|  | β-hemolysin | *hlb* | 15 (100) | 15 (100) |
| *Toxins: Exfoliative toxins* |  |  |  |  |
|  | Exfoliative toxin type c | *etc* | 15 (100) | 15 (100) |
| *Toxins: Secretion system* |  |  |  |  |
|  | Type VII secretion system | *esaA* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *esaB* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *essA* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *essB* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *essC* | 1 (6.7) | 2 (13.3) |
|  | Type VII secretion system | *esxA* | 1 (6.7) | 2 (13.3) |

4.10 Figures



Figure 4.1Example dendrogram of the RAPD fingerprints of 13 *Staphylococcus chromogenes* isolates representative of an intramammary infection (IMI) associated with a persistently high (≥200,000 cells/mL) or low (<200,000 cells/mL) somatic cell count, originating from Farm H. RAPD types were assigned an arbitrary letter based on the clustering. Seven RAPD types were identified from the 13 isolates causing IMI.



**n = 9**

**n = 11**

**n = 6**

**n = 4**

Figure 4.2Carriage of the *blaZ* gene (encoding for the production of β-lactamase) for 30 *Staphylococcus chromogenes* isolates from subclinical bovine intramammary infections (IMI) by somatic cell count (SCC) category. All isolates in the HIGH category (n = 15) come from IMI which had an SCC of ≥200,000 cells/mL associated with all quarter-day observations, and all isolates in the LOW (n = 15) category come from IMI which had an SCC of <200,000 cells/mL associated with all quarter-day observations.



Figure 4.3Distributions of putative virulence genes for 30 *Staphylococcus chromogenes* isolates from subclinical bovine intramammary infections by virulence type. Isolates of the same strain type (ST; determined by multilocus sequence typing) are listed sequentially. Scheme for grouping putative virulence genes by type adapted from Naushad et al., 2019.

CHAPTER 5: Species-specific prevalence of intramammary pathogens causing subclinical mastitis on organic dairy farms in Vermont using different facility types

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5.1 Abstract

A longitudinal study was undertaken to characterize the prevalence of intramammary infections (IMI) caused by different microorganisms on 10 small-midsize organic farms in Vermont (US), both for farms using tiestalls and farms using bedded packs. Most IMI were caused by non-*aureus* staphylococci. At the species level, *Staph. chromogenes* was the leading cause of IMI, followed by *Strep. uberis* and *Staph. aureus*. The observed diversity of species was similar to the limited research previously describing pathogen-specific prevalence of IMI on organic farms. Quarter-level prevalence of IMI by pathogen was similar between bedded pack and tiestall farms in the study.

5.2 Introduction, Methods, and Results

In May 2024, the US sold over 63 million kg of organic whole milk, a 20.2% increase from 2023 (USDA-AMS, 2024). Although ranked 19th in overall milk production, dairy farming is an incredibly important part of Vermont’s agricultural sector; dairy comprised 65% of the state’s total farm sales in 2023, the highest in the US (Progressive Dairy, 2024). In 2021 (most current USDA Certified Organic Survey), Vermont had 147 organic dairy farms, which made over 85 million kg of fluid milk, worth over $59 million (USDA, 2022).

Differences in both management practices and herd characteristics exist between organic and conventional dairies. Organic farms were found to be smaller, produce less milk, more likely to house cows in tiestalls (TS; vs. freestalls, FS), and exhibited differences in how cows were fed and watered (Zwald et al., 2004). When farms were matched for size, cows on organic farms were older, fed less grain, and produced less milk (Stiglbauer et al., 2013). Perhaps the most significant difference between conventional and organic dairies in the US is that antibiotics are not allowed for use on organic farms (USDA, 2024). Antibiotics are a significant component of mastitis control and treatment on conventional farms, leaving limited available options for organic dairy producers to effectively control mastitis (Ruegg, 2009; NMC, 2019). Although this could potentially result in worse overall udder health on organic farms vs. conventional farms, the differences between the two systems are not clear-cut. At the bulk tank milk level, organic farms were more likely to be positive for *Staphylococcus 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 (except *Staph. aureus*) differed between organic and conventional farms in the US. Overall, research suggests that differences in mastitis epidemiology may exist between conventional and organic dairy farms.

Organic dairy producers with small-midsize farms in the Northeastern US have expressed interest in bedded pack systems (BP) as an option to house cows during the non-grazing season, as these facilities integrate well with pasture-based farm systems (Andrews et al., 2021). Additionally, state and federal agencies in the US are providing financial incentives to build these structures as part of manure management practices which improve water quality and contribute to soil conservation (USDA-NRCS). Currently, most organic dairies in Vermont use a TS to house their animals while not on pasture (Andrews et al., 2021). As interest in BP grow among organic farmers, it is important to understand any udder health implications for farms using this facility type. Given the continued increase in demand for organic dairy products and the importance of organic dairies to Vermont, a longitudinal study was undertaken to describe the diversity of species causing IMI on organic dairy farms in the state. The specific objectives of the project were to characterize the prevalence of IMI caused by different microorganisms for 10 small-midsize organic farms in Vermont, both for farms using TS and farms using BP.

Enrolled farms were a non-probability subsample of certified organic dairies which had participated in previous studies and milked 35-120 cows. The study was carried out Winter 2019-2020, with 5 herds enrolled using a TS bedded with shavings/sawdust to house lactating dairy cows, and 5 herds using a BP. The inclusive term “bedded pack” encompasses both aerobically composting bedded packs and deep bedded packs, and was defined as an enclosed loose housing facility deeply bedded with organic material (Jeffrey et al., 2024). Approximately 35 cows in early- to mid-lactation were enrolled from each herd. For 8 herds with DHIA data, cows were stratified by SCC, parity, and DIM, then randomly selected across these variables. All cows were sampled in 1 herd with ~35 lactating cows, and for the remaining herd the producer generated a list of 35 cows in early lactation. Cows unable to be sampled at a follow-up visit 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).

Standard aerobic bacteriological culture of quarter-milk was performed in duplicate to identify bacterial species present according to NMC guidelines NMC, 2017). 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 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.

Isolates from both pure and mixed culture quarter-milk samples were then identified to species or genus using MALDI-TOF mass spectrometry (Microflex, Bruker Daltonics). The protocol for identifying bacterial isolates with MALDI-TOF mass spectrometry has been described previously in Haw et al. (2024). For isolates unable to be identified with MALDI-TOF, other identification methods were used (colony morphology, catalase reaction, Gram stain, PCR-based amplicon sequencing for 16S rRNA or *rpoB* gene). Using the bacteriological status and species identification, a quarter-day IMI status was assigned to each quarter observation: 1) “no growth,” when there was no significant growth; 2) “single pathogen infection,” when ≥ 100 CFU/mL of a particular pathogen was identified in pure culture on both plates (interpretation in series; Dohoo et al., 2011); 3) “mixed infection,” when ≥ 100 CFU/mL of 2 different pathogens were identified in mixed culture on both plates; and 4) “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 the IMI status was classified as no growth, single pathogen infection, or mixed infection.

Quarter-day IMI status, cow information, visit, and herd data were organized into a spreadsheet and imported into the R Statistical Programming Environment (R Development Core Team, 2023) for analysis. The quarter-level prevalence for each farm visit was calculated by dividing the number of quarters infected with a particular pathogen (or grouping of similar pathogens) by the total number of sampled quarters at risk where IMI status could be determined for that farm visit. Median and range of quarter-level prevalence for each herd was then calculated using all consecutive visits to a particular farm. Median and range of quarter-level prevalence for tiestalls and bedded packs were calculated over all 15 visits to each facility type, respectively. Overall median and range of quarter-level prevalence were calculated using all 30 visits to the 10 farms.

Median herd size was 70 lactating cows (range: 44-105) of various breeds, with a median rolling herd average of 13,250 lbs. (range: 10,675-21,204 lbs.). 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. Mean days elapsed between farm visits was 33.6 (median: 34; range: 27-43). Of the 5 enrolled BP farms, 2 were composting BP, cultivating the pack twice a day to encourage aerobic decomposition of sawdust or shavings (The Dairyland Initiative, 2024; Bewley et al., 2017). Two BP used a “traditional” or “deep bedded pack” system, where large volumes of dry straw or hay was added to bedding that accumulated over the 6-8 months cows were housed indoors (The Dairyland Initiative, 2024; Bewley et al., 2017). The remaining BP bedded with straw and woodchips and cultivated every 48 hrs., adding chopped hay and woodchips each time.

In total, 4,212 quarter-observations were collected from 1,536 quarters belonging to 384 cows were enrolled for at least 1 visit. Of these, 880 quarter-observations were excluded from further analyses: 34 did not meet definition of either having an IMI or no growth; 88 were from non-functional mammary glands; 224 were excluded due to a sampling error; and 534 were excluded because ≥ 1 of the 2 duplicate quartermilk samples was contaminated. The final data set consisted of 3,332 quarter-observations (from 1,456 quarters of 382 cows) where the IMI status of the quarter could be determined. There were 2,290 quarter-observations from no growth quarters. The mean (median; range) number of cows included per herd was 38.2 (38; 35-41), quarters per cow was 3.8 (4; 1-4) and observations per quarter was 2.3 (2; 1-4).

There were 1,042 quarter-observations from quarters with an IMI at time of sampling: 953 with an IMI due to a single pathogen (28.6% of all quarter-observations), and 89 with a mixed infection (2.7% of all quarter-observations). The quarter-level prevalence of pathogens (or grouping of similar pathogens) causing intramammary infections by farm is presented in Table 5.1. Overall, the majority of IMI were caused by all NASM species combined (median prevalence of 20%). At the species level, *Staph. chromogenes* was the leading cause of IMI (14.6%), followed by *Strep. uberis* (3.4%), *Staph. aureus* (3.2%), and *Staph. haemolyticus* (1.3%).

5.3 Discussion

*Staphylococcus* were the dominant organisms causing IMI in this population of farms, with the largest proportion of IMI caused by non-*aureus* staphylococci and mammaliicocci (NASM). The median NASM prevalence in the current study is similar to previous studies reporting a quarter-level prevalence of 26% in Canada (Condas et al., 2017) and 21% in Belgium (Valckenier et al., 2020), although higher than 1 US study (11%, Rowe et al., 2019) and lower than another Belgian study (33%; Wuytack et al., 2020). *Staph. chromogenes* was the most frequently identified species, consistent with other studies from both conventional (De Visscher et al., 2016; Condas et al., 2017; Rowe et al., 2019; Wuytack et al., 2020) and organic (Peña-Mosca et al., 2023) herds in various countries. In agreement with Peña-Mosca et al. (2023), the second most frequently isolated *Staph.* species was *Staph. aureus.* However, the quarter-level prevalence observed in the current study was much lower than the 13.6% reported for the second post-partum sampling of the 5 organic farms in Peña-Mosca et al. (2023). A similar distribution pattern of NASM was observed in both the current study and Peña-Mosca et al. (2023), where a diverse number of species were identified but the prevalence of non-*chromogenes* IMI was low. *Staph. haemolyticus* was found at almost twice the quarter-level prevalence in the current study when compared to Peña-Mosca et al. (2023) (1.3% vs. 0.7%, respectively). While Peña-Mosca et al. (2023) found *Strep. dysgalactiae* to be the dominant streptococcal species vs. *Strep. uberis* (quarter-level prevalence of 4.2% and 0.5% respectively for their second post-partum samples), the relative distribution of these 2 species was reversed in the current study (0.4% for *Strep. dysgalactiae* and 3.4% for *Strep. uberis*).

Farms from the current study exhibited a large amount of variation in quarter-level prevalence of *Corynebacterium* spp. The median prevalence in the current study (0.9%) is similar to a large US study (1.16%; Rowe et al., 2019) and lower than that reported by a large Canadian study of fresh cows (3.2% in first-calf heifers, 4.7% multiparous cows; Naqvi et al., 2018). Three farms in the current study had no *Corynebacterium* spp. isolated from subclinical IMI over all farm visits, 5 had a prevalence ranging from 0.4-4%, 1 TS had a prevalence of 7.5%, and 1 BP had a prevalence of 11.5%. Similar findings have been reported by other studies describing subclinical IMI by pathogen on organic dairies. In a comparison of 7 organic and 7 conventional herds, Mullen et al. (2013) report that percentages of quarters infected with *Corynebacterium* spp. showed high variability for the organic farms, ranging from 0 to 63.5%. Peña-Mosca et al. (2023) also found a relatively high proportion of quarters infected with *Corynebacterium* spp. (2.8-5.4% for various sampling periods post-partum). Research exploring risk factors associated with *Corynebacterium* spp. may identify whether organic farms exhibit a wider range of prevalence for these pathogens, or if this observed herd-level variability is seen in both conventional and organic farms.

Research describing the pathogen-specific prevalence of subclinical mastitis is limited for farms using BP, but Fávero et al. (2015) observed that *Corynebacterium* spp. were the most common cause of subclinical IMI in a study of 3 BP farms in Brazil, followed by coagulase-negative *Staph.* Similar to the current study, Freu et al. (2023) report *Staph. chromogenes* was the dominant cause of subclinical IMI for 7 herds using BP in Brazil. Quarter-level prevalence of *Staph. chromogenes* in that study was 24.9%, followed distantly by *Strep. agalactiae* (5.4%)and *Staph. aureus* (4.1%). For the BP in the current study, the second-most common pathogen identified was *Strep. uberis*, followed by *Staph. haemolyticus.* No *Strep. agalactiae* was found in the current study. A number of NASM species were identified which were unique to TS in the current study, including *Mammaliicoccus sciuri, Staph. auricularis, Staph. capitis, Staph. cohnii, M. fleurettii, Staph. hominis, Staph. pseudintermedius, Staph. saprophyticus,* and *M. vitilinus.* In contrast, *Staph. epidermidis, Staph. gallinarum,* and *Staph. succinus* were only isolated from IMI on BP. Work comparing NASM diversity between BP and TS is limited, but a study comparing bulk tank milk between sand-bedded FS and CBP also found that some species were unique to facility type. Adkins et al. (2022) observed a greater diversity of NASM species in bulk tank milk for FS, including *Staph. capitis, Staph. cohnii, Staph. gallinarum, Staph. hominis, Staph. hyicus,* and *Staph. succinus*, while *Staph. succinus* was the only species unique to BP.

Overall, quarter-level prevalence of IMI by pathogen was similar between BP and TS in the current study. BP systems have a number of advantages, including a smaller initial investment when compared to a new FS or TS barn (Barberg et al., 2007a; Janni et al., 2007), although the cost year-over-year for bedding is substantial (Shane et al., 2010). BP are designed for cow comfort (Barberg et al., 2007b; Bewley et al., 2012), and prevalence of lameness, foot, and leg injuries in these systems has been found to be less than TS and FS (Barberg et al., 2007b). For producers considering a transition from outdated TS barns, BP may be a viable option for dairy cattle housing in the Northeastern US. However, more research is needed in order to compare these facility types with sufficient statistical power to account for herd-level effects.

5.4 Declarations

*Ethics approval and consent to participate*

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).

*Consent for publication*

Not applicable

*Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

*Competing interests*

The authors have not stated any conflicts of interest.

*Funding*

This project was funded by the Organic Agriculture Research and Extension Initiative (OREI) from the National Institute of Food and Agriculture (USDA-NIFA grant 2018-51300-28561). The first author, Caitlin E. Jeffrey was supported by a USDA-NIFA Predoctoral Fellowship award (grant 2022-67011-36567).

*Authors' contributions*

Caitlin Jeffrey conceptualized the study, acquired funding, coordinated farm recruitment and sampling, conducted on-farm sample collection, managed and curated the data, conducted the data analysis, prepared data visualizations and presentation, wrote the original and final drafts. Pamela Adkins, conducted isolate species identification by MALDI-TOF. John Barlow conceptualized the study, acquired funding, supervised the research, conducted on-farm sample collection, reviewed and edited the manuscript.

*Acknowledgements*

The authors thank the organic dairy producers who agreed to participate in this study, for giving us their time and allowing us to collect samples from their farms. We are grateful to the numerous University of Vermont undergraduate students who assisted with sample collection. We thank the laboratory staff at the Vermont State Agricultural and Environment Laboratory for determination of somatic cell counts from the quarter milk samples. We thank Paige Isensee, Natalie Sexton, Madyson Marrs, and Allena Radford, who provided laboratory assistance in the Adkins lab at the University of Missouri.

*Authors' information*

Not applicable

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5.6 Tables

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Table 5.1 Quarter-level prevalence of pathogens (or grouping of similar pathogens) causing intramammary infections [median (range)] by farm, stratified by facility type. 3,332 quarter-level observations were collected from 1,456 quarters belonging to 382 cows during a longitudinal, cross-sectional observational study of 10 certified organic dairy farms in Vermont (US). The quarter-level prevalence represents the percent of sampled quarters infected with a particular pathogen over all sampled quarters at risk where IMI status could be determined for that farm visit. Median and range of quarter-level prevalence for each herd were calculated using all consecutive visits to a particular farm. Median and range of quarter-level prevalence for tiestalls (TS) and bedded packs (BP) were calculated over all visits to TS (n = 15) and BP (n = 15), respectively. Overall median and range of quarter-level prevalence were calculated using all visits to all 10 farms (n = 30). | | | | | | | | | | | | | | | |
|  | | | TS-1 | TS-2 | TS-3 | TS-4 | TS-5 | TS avg. | BP-1 | BP-2 | BP-3 | BP-4 | BP-5 | BP avg. | Overall |
| Num. farm visits | | | 3 | 3 | 3 | 3 | 3 | 15 | 3 | 4 | 3 | 2 | 3 | 15 | 30 |
| Pathogen (group) | | |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | *Staphylococcus aureus* | | 3.5 (3.1-4) | 1.9 (1.7-2.4) | 4 (3.4-4.1) | 3.5 (3.2-3.9) | 0.9 (0.9-1) | 3.2 (0.9-4.1) | 4.8 (4.7-8.4) | 13.1 (11.7-14.1) | 0.8 (0-2.6) | 1.3 (0.9-1.7) | 0.8 (0-0.8) | 2.6 (0-14.1) | 3.2 (0-14.1) |
|  | Non-*aureus* staphylococci and mammaliicocci | | 10 (9.7-10.4) | 19.6 (16.7-23.2) | 24 (22.3-25.6) | 15.8 (14.2-18.5) | 20.4 (19-24.5) | 19 (9.7-25.6) | 24.8 (21.9-39.8) | 14.1 (12.8-18.2) | 23.6 (21.9-25.5) | 19.4 (17.6-21.1) | 20.6 (19.4-22.4) | 21.1 (12.8-39.8) | 20 (9.7-39.8) |
|  | | *Staphylococcus agnetis* | 2 (1.8-2.1) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0.9) | 0 (0-2.1) | 2.4 (0.8-3.6) | 1.1 (0-2) | 0 (0-0) | 0.9 (0.8-0.9) | 0.8 (0.8-0.9) | 0.8 (0-3.6) | 0.8 (0-3.6) |
|  | | *Staphylococcus auricularis* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) |
|  | | *Staphylococcus capitis* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0.8 (0-0.8) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) |
|  | | *Staphylococcus chromogenes* | 6 (5.3-6.3) | 13.1 (11.7-15.9) | 16.2 (14.9-16.8) | 8.8 (7.1-8.9) | 15.5 (13.8-15.5) | 13.1 (5.3-16.8) | 15.2 (13.3-21.7) | 7.8 (6.5-10.1) | 19.5 (17.5-21.8) | 15.9 (14.3-17.4) | 16.8 (16.1-17.6) | 16.1 (6.5-21.8) | 14.6 (5.3-21.8) |
|  | | *Staphylococcus cohnii* | 0 (0-0) | 0.9 (0.8-1.2) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) |
|  | | *Staphylococcus devriesei* | 0 (0-0) | 0.9 (0.8-1.2) | 0 (0-0) | 0 (0-0) | 1.9 (1.7-2.7) | 0 (0-2.7) | 0.8 (0.8-2.4) | 1 (0-1.1) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-2.4) | 0 (0-2.7) |
|  | | *Staphylococcus epidermidis* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1) | 0 (0-1) |
|  | | *Staphylococcus equorum* | 0 (0-0) | 3.7 (1.7-3.7) | 0 (0-0) | 0 (0-0) | 1 (0.9-1.7) | 0 (0-3.7) | 0 (0-1.2) | 0 (0-1.1) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) | 0 (0-3.7) |
|  | | *Staphylococcus gallinarum* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0.8 (0-1.2) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) | 0 (0-1.2) |
|  | | *Staphylococcus haemolyticus* | 1 (0.9-1) | 0 (0-0) | 0.8 (0.8-1.7) | 1.6 (1.6-1.8) | 0 (0-0.9) | 0.9 (0-1.8) | 3.1 (2.4-4.8) | 3 (2.1-3.3) | 3.3 (2.7-3.5) | 0.9 (0.8-0.9) | 0.8 (0-1.9) | 2.7 (0-4.8) | 1.3 (0-4.8) |
|  | | *Staphylococcus hominis* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) |
|  | | *Staphylococcus hyicus* | 0 (0-0) | 0 (0-0) | 0.8 (0.8-0.9) | 0 (0-0) | 0 (0-0) | 0 (0-0.9) | 0 (0-0) | 1 (0-1.1) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.1) | 0 (0-1.1) |
|  | | *Staphylococcus pseudintermedius* | 0 (0-0) | 0.9 (0.8-1.2) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) |
|  | | *Staphylococcus saprophyticus* | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) |
|  | | *Staphylococcus simulans* | 1 (0.9-1) | 0 (0-0) | 3.2 (2.5-4.3) | 3.2 (3.1-3.5) | 0 (0-0) | 1 (0-4.3) | 2.4 (2.3-2.4) | 0 (0-0) | 0.9 (0.8-0.9) | 0.9 (0.8-0.9) | 1.5 (0.8-1.9) | 0.9 (0-2.4) | 0.9 (0-4.3) |
|  | | *Staphylococcus succinus* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1) | 0 (0-1) |
|  | | *Staphylococcus warneri* | 0 (0-0) | 0 (0-0) | 2.5 (2.4-2.6) | 1.6 (1.6-1.8) | 0 (0-0) | 0 (0-2.6) | 1.6 (0.8-2.4) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-2.4) | 0 (0-2.6) |
|  | | *Staphylococcus xylosus* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 1 (0.9-1.8) | 0 (0-1.8) | 0 (0-0) | 0.5 (0-1.1) | 0 (0-0) | 0.9 (0.8-0.9) | 0.8 (0.8-0.9) | 0 (0-1.1) | 0 (0-1.8) |
|  | | *Mammaliicoccus fleurettii* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 1 (0-1.8) | 0 (0-1.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.8) |
|  | | *Mammaliicoccus sciuri* | 0 (0-0.9) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0.9) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.9) |
|  | | *Mammaliicoccus vitilinus* | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.9) | 0 (0-0.9) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.9) |
|  | *Streptococcus dysgalactiae* | | 0 (0-0) | 3.3 (2.8-7.3) | 1.7 (0.8-1.7) | 0.8 (0.8-0.9) | 0 (0-0) | 0.8 (0-7.3) | 0 (0-0) | 1.6 (1.1-2) | 0.9 (0-1.8) | 0 (0-0) | 0 (0-0) | 0 (0-2) | 0.4 (0-7.3) |
|  | *Streptococcus uberis* | | 1 (0.9-1) | 5.6 (5-6.1) | 5 (4-5.1) | 2.4 (2.4-2.6) | 0.9 (0.9-1) | 2.4 (0.9-6.1) | 3.6 (3.2-5.5) | 4.2 (1.1-6.1) | 2.4 (1.8-3.5) | 0.4 (0-0.8) | 5.3 (4-5.6) | 3.6 (0-6.1) | 3.4 (0-6.1) |
|  | *Aerococcus* spp.1 | | 1 (0-1.8) | 0 (0-1.7) | 0 (0-1.7) | 0 (0-1.6) | 0 (0-0.9) | 0 (0-1.8) | 0 (0-4.8) | 0.5 (0-4) | 0 (0-0.9) | 0 (0-0) | 1.6 (0-3.7) | 0 (0-4.8) | 0 (0-4.8) |
|  | Other streptococcal and streptococcal-like organisms2 | | 0 (0-0) | 0 (0-0.8) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-1.2) | 0.5 (0-1.1) | 0 (0-1.6) | 0 (0-0) | 0.8 (0-1.6) | 0 (0-1.6) | 0 (0-1.6) |
|  | *Corynebacterium* spp.3 | | 4 (0.9-6.3) | 7.5 (6.7-11) | 2.4 (0.8-2.6) | 1.6 (0-1.6) | 0 (0-0.9) | 1.6 (0-11) | 0.8 (0-8.4) | 11.5 (5.3-20.2) | 0 (0-0) | 0.4 (0-0.8) | 0 (0-0) | 0 (0-20.2) | 0.9 (0-20.2) |
|  | *Kocuria* spp.4 | | 1 (0-3.5) | 0 (0-0.8) | 1.6 (0-2.6) | 0 (0-1.6) | 0 (0-0.9) | 0 (0-3.5) | 0 (0-1.6) | 0 (0-0) | 0 (0-2.6) | 0 (0-0) | 0 (0-0) | 0 (0-2.6) | 0 (0-3.5) |
|  | Other gram-positive bacteria | | 1 (0.9-1) | 0 (0-1.2) | 0.8 (0-0.8) | 0.8 (0-1.6) | 0 (0-1.8) | 0.8 (0-1.8) | 0.8 (0-0.8) | 2.1 (1-4.3) | 0 (0-0) | 1.3 (0.8-1.8) | 0.8 (0-0.8) | 0.8 (0-4.3) | 0.8 (0-4.3) |
|  | Coliforms5 | | 0 (0-0) | 0 (0-0.9) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.9) | 0 (0-0) | 1.1 (1-2) | 1.8 (0-2.4) | 0.5 (0-0.9) | 0 (0-0) | 0 (0-2.4) | 0 (0-2.4) |
|  | Other gram-negative bacteria | | 0.9 (0-2) | 0.9 (0.8-1.2) | 0 (0-0) | 0.8 (0-0.8) | 0.9 (0-0.9) | 0.8 (0-2) | 0 (0-0) | 0.5 (0-2) | 0 (0-0.9) | 0.8 (0-1.7) | 0 (0-0.9) | 0 (0-2) | 0 (0-2) |
|  | *Candida rugosa* | | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-1.2) | 0 (0-1.2) |
|  | Unable to be identified | | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0.8) |
| 1 *Aerococcus* sp. (genus-level identification only), *Aerococcus viridans* | | | | | | | | | | | | | | | |
| 2Other streptococcal and streptococcal-like organisms not listed separately: *Streptococcus* sp*.* (genus-level identification only)*, Streptococcus canis, Enterococcus saccharolyticus* | | | | | | | | | | | | | | | |
| 3 *Corynebacterium* sp. (genus-level identification only), *C. amycolatum, C. callunae, C. casei, C. confusum, C. glutamicum, C. stationis, C. ulcerans, C. variabile, C. xerosis* | | | | | | | | | | | | | | | |
| 4 *Kocuria* sp. (genus-level identification only), *Kocuria* *carniphila, Kocuria* *palustris* | | | | | | | | | | | | | | | |
| 5 *Enterobacter* sp. (genus-level identification only), *Escherichia coli, Klebsiella aerogenes, Klebsiella pneumoniae, Klebsiella variicola, Serratia marcescens* | | | | | | | | | | | | | | | |

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 intramammary infections (IMI) in organic dairy cows in Vermont. In particular, this work generated foundational knowledge about staphylococcal species causing mastitis on organic dairies. Bacteria belonging to the genus *Staphylococcus* are the predominant pathogens causing IMI in dairy animals, both on organic farms in Vermont and across the globe.

Chapter 1 of this dissertation described the findings of an observational study of organic dairies 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 more commonly used facility types.

The diversity of bacterial species causing IMI on 10 organic farms in Vermont were then identified in a longitudinal study, for all types of pathogen in Chapter 5 and specifically staphylococci and mammaliicocci in Chapter 3. An even number of organic herds using bedded packs and tiestall barns were enrolled in this study. Species belonging to the genus *Staphylococcus* were primarily responsible for causing 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 found for 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 *Staph. 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 that previously reported for conventional herds. Compared to no growth quarters, SCC was higher in quarters infected for 9 of 10 staphylococcal species identified. Although the 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 no growth 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 *Staph. chromogenes* causing persistent 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 (ST) of *Staph. chromogenes* were not found to be associated with either SCC category. Ten different ST were identified, 4 of which were newly-described. The only antimicrobial resistance gene (ARG) 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 3 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 each month, cows with chronic IMI month to month, proportion of cows with an elevated SCC, and all bulk tank SCC were 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 virulence factors 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 SCC 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 virulence factors stratified by type of virulence category, the presence of each additional 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 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 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 this methodology was 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. Identifying more isolates by MLST and screening for *coa* could determine if carriage of this notable staphylococcal virulence factor was more likely for isolates belonging to this particular 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.*

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

Ten of the 30 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 8 isolates negative for *blaZ* based on whole genome sequencing were susceptible to penicillin *in vitro,* while the 4 *blaZ-*positive isolates were determined to be resistant. Although no other ARG 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 ARG are only looking for previously-described genes and mechanisms of resistance in bacteria; the process of identifying novel mechanisms of resistance 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 *Staph. 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 4 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 4 was from ST6, 1 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*. 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 genes (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.

6.2.4 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., 2023b). 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. (2023b). 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. (2023b), 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, MALDI-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. 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 3 isolates are all from the same farm, and 2 were isolated from the same quarter over sequential visits. It is interesting to consider why these 3 particular *Staph. chromogenes* isolates out almost 500 were unable to be identified with MALDI-TOF. Unfortunately, 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 others within *Staph. chromogenes,* preventing them from being identified in the same manner. Further, if this was the case, it may lead to the question of whether MALDI-TOF could 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 used 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 ARG has been demonstrated for bovine *Staph. aureus.* Evidence for association between ST and virulence potential, as well as ST and carriage of ARG, was observed in Persson Waller et al. (2023b) for *Staph. chromogenes.* When Naushad et al. (2019) applied various clustering approaches in order to determine whether particular virulence factor 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 appears that the pattern of virulence genes carried by NASM isolates is also likely species-dependent. Therefore, this genetic basis for carriage of virulence factors and ARG 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, 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 collected from a greater number of farms and the ability to sequence more *Staph. chromogenes* isolates may be better able to explore associations of ARG and virulence factor carriage by ST (as determined by MLST). Larger sample sizes would likely result in a greater diversity of ST, with more isolates assigned to each 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 ARG would predict if a *Staph. chromogenes* isolate would be associated with a persistently high or low SCC IMI, it may be interesting to these attributes between isolates associated with clinical vs. non clinical mastitis. In a study of 217 *Staph. aureus* IMI isolates typed using pulsed-field gel electrophoresis, 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 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.

6.2.5 Further work to better understand differences between organic and conventional dairy farm systems

While Chapters 3-5 described the species-specific prevalence of IMI pathogens, as well as the virulence potential and ARG carriage of *Staph. chromogenes* from organic dairy farms, the longitudinal study described was not designed to identify differences between conventional and organic dairy farms. Although Chapters 3 and 5 found that the species diversity and prevalence of various NASM were similar to what has previously been described for conventional farms (and 1 study of organic farms), it would be interesting to carry out research directly comparing NASM causing IMI in both types of system. Research summarized in Chapter 1 compared prevalence of IMI due to “coagulase negative staphylococci” generally, but these isolates were not identified to species level. Differences in management practices, facility types, and cow demographics between conventional and organic dairies was summarized in Chapter 5. Given that variation in NASM species distribution and diversity is associated with a variety of herd and cow-level effects, it is possible that these differences may lead to dissimilar relative distributions or diversity of NASM causing IMI between the two farm types. Furthermore, although comparison of resistance profiles of staphylococci causing IMI between organic and conventional dairies has generally has been well-described (Chapter 1), these studies are again limited by a lack of identification to species level. As antimicrobial resistance (AMR) patterns are species-specific, potential exists for research comparing the both genotypic and phenotypic species-specific resistance profiles for NASM causing IMI on organic and conventional dairies. Exploring this topic would identify what kind of resistance was prevalent in NASM not experiencing selective pressure from antibiotic usage, which would be the first step in understanding why resistance is maintained in these populations, what type of resistance is prevalent.

Related to this, potential exists for research comparing the most common ST of *Staph. chromogenes* causing IMI on organic and conventional farms. Comparison of predominant ST within a given species causing IMI could further our understanding of the complex interplay between phylogeny and selection pressures resulting from management factors on the resistance profiles of these mastitis pathogens. Previous findings suggests that ST may vary between organic and conventional farms, although researchers were studying fecal *E. coli* and not mastitis pathogens. Walk et al. (2007) found that phylogenetic groupings varied between organic and conventional dairies, suggesting there may be differences between lineages of *E. coli* in their ability or likelihood of acquiring resistance genes. Based on their findings, the authors conclude that “organic farming practices not only change the frequency of resistant strains but also impact the overall population genetic composition of the resident *E. coli*flora.”

A few studies describe resistance patterns of mastitis pathogens before and after transitioning to organic status, and these are summarized in Chapter 1. However, most were (understandably) limited in both the number of herds enrolled and the amount of time farms were followed. Although likely logistically difficult and expensive, a long-term study of farms transitioning from conventional to organic status would be incredibly valuable in understanding what types of AMR is maintained in organic dairy herds and for how long. This research could help us start understanding where the resistance genes in these bacteria came from, how long they maintain them in the absence of selective antimicrobial pressure, and specifically what types of resistance are maintained.

An extremely unique opportunity exists for research using IMI isolates from the longitudinal study described in Chapters 3-5. Staphylococci causing IMI from a particular farm enrolled in the current study were collected and stored from a previous study, approximately 25 years earlier. Identifying these isolates to the species level and then comparing resistance profiles across 25 years could give insight into trends in AMR prevalence over time. This particular farm was founded in the 1970s and has no history of antibiotic use. The 3 *Staph. chromogenes* isolates included from this farm in Chapter 4 (assemblies 1-3, Table 6.1) were found to be penicillin susceptible and *blaZ-*negative. However, 19 *Staph. aureus* and 92 NASM isolates belonging to 8 different species (including 50 additional *Staph. chromogenes*) have been identified and stored from this farm in the current study. This collection of historical and new isolates from the same farm held by our research group would be limited to a case study. However, potential exists for collaboration with other research groups who may happen to have a similar collection of isolates. A study like this may help shed light on what different types of resistance persist long-term among mastitis pathogens in a dairy farm environment in the absence of antimicrobial use. As summarized in Chapter 1, some degree of AMR was found in isolates despite decreased (EU) or absence (US) of selective pressure of antimicrobial use. Additionally, isolate collections like this would allow for comparing what version of an ARG was present in bacteria at different points in time. Specifically, one could compare type of carriage of *blaZ* (chromosomally vs. plasmid) between old and new isolates, or the particular protein signature of the β-lactamase enzyme encoded by *blaZ*. One could then determine whether the same type of penicillin resistance has been maintained in staphylococci for a particular farm, or if the predominant ARG conferring resistance to penicillin has shifted over time. Assuming there is a fitness cost to bacteria for maintaining AMR genes (Vanacker et al., 2023), this certainly begs the question of why resistance genes have been maintained to any degree in the absence of selective antimicrobial pressures. If particular ARG were shown to be maintained in mastitis pathogens from organic farms for 25 years, identifying where these ARG are carried in bacterial genomes could help determine why they have been maintained. Are these ARG close to genes associated with processes necessary for survival, and therefore get “carried along?” Or, are they linked to a different trait which is locally advantageous to survival in that particular organic dairy farm? Improving our understanding of how AMR is transmitted or retained in dairy farm systems is essential for developing effective strategies to mitigate resistance among mastitis pathogens.

6.3 General conclusions and overall significance

The research presented in this dissertation indicates that the species distribution and prevalence of staphylococcal species causing IMI on organic dairy farms in Vermont are similar to what has been described previously for conventional farms.Furthermore, the inflammatory reaction in the mammary gland associated with IMI due to different staphylococcal species is also similar to that which has been previously described. The impact of IMI caused by staphylococci seems to be consistent across differing facility types used by organic farms, as shown by comparison of bulk tank milk data presented in Chapter 2 and the diversity and relative proportion of species causing IMI at the quarter-level presented in Chapter 5.

This work underscores that NASM are indeed worthy of consideration as significant mastitis pathogens, and are not “minor” mastitis pathogens as some have previously described them. As a group, NASM were clearly the predominant pathogen causing IMI, with a 20% median quarter-level prevalence described in Chapter 5. Nine of the ten species analyzed in Chapter 3 species elevated quarter SCC above no growth quarters. Although this increase in quarter SCC was modest for most species observed, their widespread nature can still result in an increased bulk tank SCC. This work also underscores the importance of *Staph. chromogenes* specifically as a mastitis pathogen. I found a prevalence of 33% prevalence penicillin resistance in *Staph. chromogenes*, which is especially noteworthy as these isolates originate from organic farms. Findings from our longitudinal study show that this mastitis pathogen has a great ability to persist in the mammary gland, as well as cause a number of infections associated with a persistently high SCC. Furthermore, findings from this work also suggest that particular strains of *Staph. chromogenes* behave in a contagious manner within a herd, as the same RAPD type was found causing IMI in multiple animals on a farm, and particular strains as identified by MLST appear to be dominant at different farms.

This work contributes to contributes to the understanding of intraspecies diversity within *Staph. chromogenes,* the leading cause of IMI in dairy animals worldwide. Four novel ST were identified using MLST, and 10 MLST were identified to be causing 30 persistent IMI. The AMR and virulence profile for 30 *Staph. chromogenes* were described from whole genome sequence data, with the identification of two virulence genes not widely reported for bacteria of this species (*coa*, encoding staphylocoagulase, and *etc,* encoding exfoliative toxin type C). Although no association was found between virulence and AMR carriage and whether or not the associated persistent IMI would have a high or low SCC, findings seem to indicate to a genetic basis for virulence and AMR carriage in *Staph. chromogenes.*

Taken together, my research generated foundational knowledge about the epidemiology of staphylococci causing mastitis on organic dairy farms in Vermont. While all dairy producers rely on best management practices to support cow health, mastitis control is of the utmost importance in the prevention of IMI on organic farms. Better understanding the epidemiology of mastitis pathogens leads to more effective measures which prevent or limit transmission of IMI. Mitigating the effect of mastitis caused by staphylococci through more effective, targeted prevention and control measures helps dairy producers achieve quality price premiums and results in a higher-quality product for consumers.

6.4 Tables

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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** | - |

comprehensive bibliogrpahy

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