Non-*aureus Staphylococci* and related *Mammaliicoccal* species and their association with udder health in organic dairy cows

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Restrictions placed on antimicrobial use lead to important challenges for dairy farms to
maintain udder health, which are especially challenging for organic dairy farmers. This
dissertation aims to provide additional understanding of the epidemiology of mastitis in organic
dairy cows and the potential of Non-*aureus Staphylococci* and related *Mammaliicoccal* species
(**NASM**) to control mastitis on dairy farms. Multiple investigations were performed to: explore
the intramammary infections (**IMI**) dynamics on organic dairies, investigate the relationship
between IMI, udder health, and milk production and investigate the antimicrobial activity of
NASM against mastitis pathogens.

In the first and second chapter, an overview of mastitis significance and the main mastitis
control strategies was provided. In these chapters, NASM epidemiology and potential probiotic
properties of these microorganisms was described. The third chapter provides a description of
IMI prevalence and persistence in first-lactation cows across 5 different organic dairy farms and
showed a high prevalence at calving and persistence of IMI by *Staphylococcus aureus* and
Streptococcus spp., two major mastitis pathogens. In the fourth chapter, we investigated the
association between the presence and persistence of IMI in the first 35 DIM, udder health, and
milk production in the first 180 days in milk (**DIM**). Our findings revealed that IMI caused by
Staphylococcus aureus and *Streptococcus* spp., led to an increased SCC and, in the case of
Streptococcus spp., a decreased in milk production. Our findings also suggested an association

between IMI persistence and the number of times that a cow had high SCC throughout the first 180 DIM. The fifth chapter investigated the relationship between NASM *in vitro* inhibitory activity and the presence of IMI, encountering that the presence of high *in vitro* inhibitory activity was associated with a lower risk of IMI. The last experimental chapter (sixth chapter) had the main objective of investigating the genome of NASM isolates with a focus on their phylogeny, the presence of genes related to the production of antimicrobial peptides (**AMPs**), and *in vitro* antimicrobial activity. We encountered a high prevalence of genes related to the production of AMPs. However, their presence was not associated with the *in vitro* antimicrobial activity of NASM isolates. NASM *in vitro* antibacterial activity was not related to clade membership, apart from isolates classified as *Staphylococcus succinus*.

This Ph.D. dissertation provides substantial knowledge about udder health on organic dairy farms and initial steps into the potential utilization of NASM to control mastitis in dairy farms. Nonetheless, further knowledge is needed to investigate the mechanisms leading to the antimicrobial activity of NASM against mastitis pathogens.

Future studies should focus on identifying the mechanisms behind the antimicrobial properties of NASM and investigate if the inoculation of NASM isolates with high antimicrobial activity and/or their AMPs leads to a reduced risk of IMI and mastitis.

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CHAPTER 1: INTRODUCTION

Mastitis is the most prevalent disease in dairy farms and the main reason for administration of antimicrobials (USDA, 2016). This disease causes important economic losses to the dairy industry associated not only to the discarded milk and additional expenses for mastitis treatment, but also to a reduced milk production of mastitic cows, reduced longevity and impaired reproductive efficiency (Rollin et al., 2015; Aghamohammadi et al., 2018).

Mastitis has a complex epidemiology in which mastitis pathogens can be identified in the mammary gland of dairy cows, but also from the environment or other body sites (Zadoks et al., 2011). These microorganisms could lead to IMI in dairy cows during the milking procedure (e.g., through milking equipment or milkers' hands) (Barkema et al., 2009). This is commonly referred to as a contagious epidemiology (Barkema et al., 2009). On the other hand, environmental mastitis pathogens cause an ascendant infection of the mammary gland between milkings or during the dry period (e.g., through bacteria present on the bedding, farm tracks, or even in the pasture) (Lopez-Benavides et al., 2007; Klaas and Zadoks, 2018).

Mastitis pathogens also show a varying impact on udder health in which some microorganisms are frequently associated with a substantial impairment of udder health (.e.g., major mastitis pathogens) (Keane, 2019). These pathogens include *Staphylococcus aureus*, *Streptococcus* spp. and coliforms (Keane, 2019). On the other hand, minor pathogens have little or no impact on udder health and include Non *aureus Staphylococci* and closely related *Mammaliicoccal* (**NASM**) and *Corynebacterium bovis* (Reyher et al., 2012). The adaptation of these microorganisms to the mammary gland also shows wide variations (Contreras and Rodríguez, 2011; Zadoks et al., 2011). Some of the mastitis pathogens show little adaptation to the mammary gland and lead to short-lasting IMI. For instance, most gram-negative

microorganisms with the exception of *Klebsiella* show usually a low adaptation to the mammary gland (Klaas and Zadoks, 2018). Other microorganisms possess multiple mechanisms to survive in the mammary gland, including evading or modulating immune response and/or forming abscesses in mammary tissue (Rainard et al., 2018; Fergestad et al., 2021; Kabelitz et al., 2021). These microorganisms can cause infections that can persist in the mammary gland for long periods and can even be carried across lactations (Veh et al., 2015).

This explains that while preventive measures are essential for mastitis control, antibiotic therapy remains crucial for controlling mastitis (Ruegg, 2017). In the last years, there has been an increasing pressure in livestock production for reducing the use of antimicrobials in livestock production (Krömker and Leimbach, 2017). Multiple measures can be used to achieve this objective such as the use of selective treatment of clinical mastitis cases using on-farm culture or the use of selective dry cow therapy (McCubbin et al., 2022; de Jong et al., 2023). A step beyond this is the transition from conventional to organic farming (NMC, 2019). Organic dairy farms are under the regulation of National Organic Program (USDA, 2023), that restricts the use of antibiotics in dairy farms. This restriction, considering the important role of antibiotics on mastitis control plans (Winder et al., 2019; McCubbin et al., 2022), could have an impact on udder health, especially for those microorganisms well adapted to the mammary gland. In fact, previous research has shown a higher prevalence of *Staphylococcus aureus* on organic compared to conventional dairy farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). Nonetheless, knowledge about udder health in organic dairy farms is still limited, with little knowledge about the distribution and persistence of mastitis pathogens in organic dairy farms. In addition, it is possible that restrictions imposed on farms under organic management may increase the capacity

of mastitis bacteria to persist within the mammary gland. This has the potential to amplify the negative impacts on udder health, especially for udder-adapted microorganisms. As a result, more research into this subject is required to understand the potential impacts of organic management on IMI dynamics and udder health.

Multiple alternatives to antibiotics have been investigated for their use in dairy farms including the use of homeopathy (Hektoen et al., 2004; Mathie and Clausen, 2015), phytoterapeutics (Mullen et al., 2018), phage therapy (Dias et al., 2013) or the use of bacterial antimicrobial peptides (**AMPs**) (Nascimento et al., 2005; Newstead et al., 2020) with limited success and no commercially available product.

Non *aureus Staphylococci* and closely related *Mammaliicoccal* species are a diverse group of microorganisms that are the most prevalent microorganisms leading to IMI on conventional dairy farms (De Buck et al., 2021). Infusion of NASM into the mammary gland has been related to a reduced risk of *Staphylococcus aureus* IMI (Reyher et al., 2012). In addition, some studies have shown that cows with NASM IMI have reduced incidence of clinical mastitis and higher milk production in the following lactation (Compton et al., 2007a; Piepers et al., 2010, 2013). A few questions that remain to be answered are: what is leading to this protective activity? Is NASM protective activity driven by the production of AMPs or something else? In addition, if it is actually driven by AMPs, which AMPs are responsible for these protective effects?

In an effort to address these questions, prior studies have investigated NASM *in vitro* antimicrobial activity, showing that NASM can inhibit the growth of *Staphylococcus aureus* in vitro (De Vliegher et al., 2004c; Braem et al., 2014; Carson et al., 2017; Toledo-Silva et al.,

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2022). In human studies, in vitro antimicrobial activity of these microorganisms have been related to the production of AMPs (Nakatsuji et al., 2017). However, it is still not clear which specific species or strains are related to *in vivo* protective effects. In addition, most of these studies used the cross-streak method that only provides a qualitative estimation of the *in vitro* antimicrobial activity (De Vliegher et al., 2004c; Braem et al., 2014; Carson et al., 2017). Agar dilution methods provide a quantitative estimation of *in vitro* antimicrobial activity but have only been investigated in a small number of isolates containing a few species (Toledo-Silva et al., 2022). Hence, an investigation of agar dilution methods with a larger number of species is needed to further investigate the in vitro inhibitory activity of NASM against mastitis pathogens. Lastly, it is vital to understand the impact on udder health of specific species or strains within this group.

It is also very important to further elucidate the virulence potential and ability to carry antimicrobial resistance genes of these microorganisms. However, the genomes of NASM isolates have only been investigated in a few studies using whole genome sequencing (Naushad et al., 2016, 2019; Fergestad et al., 2021). These studies were carried out in NASM isolates harbored from conventionally managed dairy cows. Hence, it is plausible that the virulence or prevalence of antimicrobial resistance genes may be different in those from organic dairy farms, and further research on this topic is needed.

The main goal of this project is to contribute to our understanding of the relationship between commensal and pathogenic bacteria isolated from the mammary gland. We expect that our project will improve our understanding of the potential protective effects of NASM against infection of the mammary gland by major pathogens such as *Staphylococcus aureus* and

Streptococcus spp., and the possible mechanisms involved in NASM protective activity.

Additionally, the potential identification of NASM strains on teat-apex with protective activity

and their AMPs by this project could then be further investigated for the prospective

development of products to treat or prevent mastitis in dairy farms.

CHAPTER 2: LITERATURE REVIEW

2.1. INTRODUCTION

Mastitis is defined as the inflammation of the mammary gland and is one of the most important and prevalent diseases that affect dairy cows (Ruegg, 2017). This inflammatory disease is caused by intramammary infections (**IMI**) and is highly prevalent in dairy production systems, including confinement and grazing systems (Washburn et al., 2002; Zadoks and Fitzpatrick, 2009). Mastitis is most commonly found in its subclinical presentation, when an ascending infection of the mammary gland by mastitis pathogens leads to an elevated somatic cell count (**SCC**) without any macroscopic changes in the appearance of the milk (Adkins and Middleton, 2018). In other instances, animals could show signs of clinical mastitis, such as the presence of abnormal milk, swollen mammary quarters, and/or general symptoms (Adkins and Middleton, 2018; Ruegg, 2021). In 2014, it was estimated that 24.8% of cows in the United States had at least one event of clinical mastitis during lactation, making mastitis the primary reason for administering antibiotics to dairy cattle in conventional dairy farms (USDA, 2016). Additionally, it was determined that 93% of cows in the United States receive antibiotic treatment at dry-off as a way to treat and prevent IMI during the dry period (USDA, 2016). The following review will discuss the importance of mastitis on dairy farms, with an emphasis on the challenges faced by organic dairies, and potential alternatives for its control.

2.2. MASTITIS IMPORTANCE ON DAIRY FARMS

Mastitis has been linked to significant economic losses that impact the profitability of dairy farms (Halasa et al., 2007). Studies have demonstrated an association between mastitis and reduced milk production (Gröhn et al., 2004; Hand et al., 2012; Hadrich et al., 2018) and changes

in milk components (Gonçalves et al., 2020). These effects can vary depending on the specific mastitis pathogen involved (Gröhn et al., 2004; Gonçalves et al., 2020). The reduction in milk yield can be related to the direct impact of mastitis microorganisms on mammary gland tissue (Enger et al., 2020) or it may also occur indirectly through the development of an inflammatory response (Sordillo and Mavangira, 2014). Economic losses due to clinical mastitis in the United States, have been estimated at $444 per case (Rollin et al., 2015). A significant proportion of these losses (28%) are related to a reduction in future milk production (Rollin et al., 2015). Another substantial portion of these losses (41%) is attributed to the premature culling of cows with mastitis (Rollin et al., 2015; Hertl et al., 2018). Indeed mastitis is an important reason for culling cows in the United States (USDA, 2016). Subclinical mastitis is also a significant contributor to economic losses (Hand et al., 2012; Hadrich et al., 2018). In a prior North American study, monthly DHIA records from 541,594 cows across 11,740 farms were analyzed for 10 months. The study observed that milk yield loss increased as the number of test days with SCC ≥100,000 cells/mL increased, with daily losses ranging from $1.20 per cow per day in the first month to $2.06 per cow per day in the 10th month (Hadrich et al., 2018).

Mastitis has also been related to impaired animal welfare, due to the presence of signs of animal discomfort (Stangaferro et al., 2016; Petersson-Wolfe et al., 2018; Rial et al., 2023). For instance, prior studies showed that cows with clinical mastitis had decreased dry matter intake and altered feeding behavior, spending more time standing and less time self-grooming (Sepúlveda-Varas et al., 2013).

Reproductive efficiency can be compromised during mastitis through the release of inflammatory cytokines and prostaglandins that impair reproductive function (Wang et al.,

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2021). A prior study demonstrated that cows with clinical mastitis took longer to get pregnant (20 days longer) compared to cows without clinical mastitis (Santos et al., 2004). This effect was attributed to changes in conception and pregnancy rates. This association was particularly notable when mastitis occurred between the first artificial insemination and pregnancy diagnosis (Santos et al., 2004). Furthermore, a previous study also demonstrated that this association varied depending on the microorganism responsible for the clinical mastitis case. The negative effect was more pronounced for *Staphylococcus aureus*, *Streptococcus* spp., coliforms and *Mycoplasma* spp., while it was not evident for clinical mastitis caused by non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (**NASM**) and *Corynebacterium bovis* (Dalanezi et al., 2020). High SCC (often used to define subclinical mastitis) has also been associated with impaired reproductive performance, especially when it occurs between 3 days before to 32 days after the first artificial insemination (Fuenzalida et al., 2015). Although a meta-analysis (Dolecheck et al., 2019) confirmed the overall negative impact of mastitis on reproductive performance in cows, it also revealed significant variability across studies, suggesting that the extent of this impact is influenced by the timing of mastitis occurrence (i.e., pre vs. post artificial insemination) and type of mastitis (i.e., subclinical vs. clinical mastitis). The result of this meta-analysis demonstrated that, on average, cows experiencing mastitis require an additional 9 days for their first service, 23 days to achieve pregnancy, and an additional 0.50 services per conception compared to cows that did not experience mastitis (Dolecheck et al., 2019). The economic losses of clinical mastitis associated with a compromised reproductive function were estimated at 8% of total losses attributed to clinical mastitis (Rollin et al., 2015).

The points discussed above emphasize the importance of mastitis on dairy farms and highlight the need for effective control and prevention strategies to reduce the impacts of mastitis.

2.3. MASTITIS EPIDEMIOLOGY

Mastitis is a multifactorial disease in which factors related to the cow, environment, management, and the presence of mastitis-causing pathogens have a significant impact on the risk of an IMI. Indeed, mastitis could be represented as a continuous battle between the cow's immune system and the bacterial load of mastitis pathogens challenging the mammary gland. The epidemiology of these microorganisms is complex with pathogens being found in the environment, the mammary gland of other dairy cows, or in some cases even other body sites (Zadoks et al., 2011).

2.3.1. Pathogens: Mastitis pathogens

Mastitis pathogens are frequently classified based on their epidemiology as contagious or environmental (Contreras and Rodríguez, 2011). The transmission of contagious mastitis pathogens occurs predominantly during the milking process through the milking machine and milkers' hands (Barkema et al., 2009). On the contrary, environmental pathogens multiply and survive in the environment are typically trasmitted between milkings and during the dry period (Klaas and Zadoks, 2018). These microorganisms can be identified in the bedding material, pasture, and farm tracks (Lopez-Benavides et al., 2007; Klaas and Zadoks, 2018).

However, with the advent of molecular techniques, it has become evident that the delineation between contagious and environmental pathogens is not always clear, and there can

be significant variations in the epidemiology of mastitis-causing species within each category (Zadoks et al., 2011; Woudstra et al., 2023). For instance, *Streptococcus agalactiae* and *Staphylococcus aureus* are microorganisms highly adapted to the mammary gland that frequently lead to persistent IMI (Keefe, 1997; Bardiau et al., 2014) and have been classified as contagious microorganisms (Keefe, 2012). Nonetheless, it has been shown that there are strains of *Staphylococcus aureus* (Rainard et al., 2018) and *Streptococcus agalactiae* (Jørgensen et al., 2016) that exhibit evidence of an environmental transmission. Similarly, *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**) and gram-negative bacteria have predominantly been classified as environmental pathogens (Kabelitz et al., 2021). However, strain-typing studies have suggested that the use of this classification may be misleading (Woudstra et al., 2023). For instance, *Streptococcus dysgalactiae* has been classified as an "intermediate" pathogen, in which strains show a divergent epidemiology (Wente and Krömker, 2020; Kabelitz et al., 2021). In contrast, *Streptococcus uberis* is an important mastitis pathogen in grazing systems (Compton et al., 2007a; McDougall et al., 2007), that frequently leads to short-lasting (i.e., transient) IMI with an environmental transmission (Lopez-Benavides et al., 2007; Wente et al., 2019; Leelahapongsathon et al., 2020). However, reports that show evidence of contagious epidemiology do exist (Davies et al., 2016; Woudstra et al., 2023). This finding was supported by the fact that a few strains were responsible for an important proportion of the *Streptococcus uberis* IMI in different studies (Davies et al., 2016; Woudstra et al., 2023). While this underscores the significance of strain-specific considerations when investigation the route of transmission of mastitis pathogens, it is also essential to recognize that the impact of IMI on udder health varies among different mastitis pathogens (Gröhn et al., 2004; Rowe et al., 2021).

Mastitis pathogens are commonly categorized into two groups according to their impact on udder health: major mastitis pathogens, which exert a significant negative impact on udder health (Keane, 2019), and minor mastitis pathogens, which typically cause mild or no detrimental effects on udder health (Reyher et al., 2012).

Microorganisms commonly classified as major mastitis pathogens include *Staphylococcus aureus*, SSLO and coliforms (Zadoks et al., 2011; Keane, 2019). Most studies have shown an increased SCC and risk of clinical mastitis in cows infected by major mastitis pathogens (Compton et al., 2007a; Paradis et al., 2010; Piepers et al., 2010; Valckenier et al., 2021). The majority of these research investigations also documented a reduced milk production in cows with major mastitis pathogens IMI (Piepers et al., 2010; Valckenier et al., 2021), although contradictory reports exist. For instance, one observational study reported no association between the presence of *Staphylococcus aureus* IMI and milk production (Paradis et al., 2010). Likewise, another observational study did not report an association between the presence of major mastitis pathogens IMI and milk production (Compton et al., 2007a).

Conversely, NASM and *Corynebacterium bovis* are commonly referred to as minor mastitis pathogens (Reyher et al., 2012; De Buck et al., 2021). These microorganisms usually show a small or negligible impact on udder health, in which most studies report an increased SCC (Paradis et al., 2010; Piepers et al., 2010; Fry et al., 2014; Tomazi et al., 2015; Nyman et al., 2018; Valckenier et al., 2020). However, one study showed no association between presence of NASM IMI and SCC (Compton et al., 2007a). Likewise, NASM impact on milk production has yielded inconsistent results across different studies. Some studies have reported a higher milk production in cows with NASM IMI (Compton et al., 2007a; Piepers et al., 2010, 2013), while in

others showed no association (Paradis et al., 2010; Tomazi et al., 2015; Heikkilä et al., 2018; Nyman et al., 2018; Valckenier et al., 2020). Many of these inconsistencies regarding the pathogenicity of NASM could be attributed to the fact that this diverse group is composed of more than 50 different species (De Buck et al., 2021). Prior data have shown that the pathogenicity of NASM differs across different species and their distributions are herd specific (De Buck et al., 2021). Some studies have suggested that special attention should be given to *Staphylococcus chromogenes, Staphylococcus simulans,* and *Staphylococcus xylosus* due to their relationship with an increased SCC (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2016a). However, other studies have shown that IMI caused by some other NASM species may also be related to a higher risk of high SCC, indicating that variation in the pathogenicity within species does exist (Condas et al., 2017; Taponen et al., 2022).

The adaptation of bacterial pathogens within the udder, and consequently, their ability to lead to persistent IMI, also differs across NASM species (De Visscher et al., 2014; Jenkins et al., 2019; Wuytack et al., 2020). *Staphylococcus chromogenes* is the NASM with the highest prevalence across multiple studies (De Buck et al., 2021), and is frequently associated with persistent IMI (Fry et al., 2014; Jenkins et al., 2019; Valckenier et al., 2021). Other NASM species have been frequently isolated in the environment such as *Staphylococcus cohnii*, *Staphylococcus fleurettii*, *Staphylococcus equorum*, *Staphylococcus haemolyticus,* and *Staphylococcus sciuri* (De Visscher et al., 2014; Jenkins et al., 2019). With these latter microorganisms exhibiting a low prevalence of IMI that varies across different herds and studies (De Buck et al., 2021).

2.3.2. Host: Mammary gland immune system

The mammary gland immune system includes physical barriers that impede the invasion of pathogens through the teat canal, such as the teat end sphincter and the keratin plug (Rainard and Riollet, 2006). The teat end sphincter has muscles surrounding the teat canal to prevent bacterial penetration (Sordillo, 2018). In addition, the keratin plug seals the mammary gland between milkings and during the dry period to prevent ascending infections by microorganisms (Rainard and Riollet, 2006; Sordillo, 2018). Keratin can trap bacteria and possesses antibacterial properties, making it an important component of the mammary gland immune system (Rainard and Riollet, 2006; Sordillo, 2018).

The udder immune system also contains multiple humoral components that play an important role in the innate immune response. These components, including complement, lactoferrin, transferrin, lysozyme, lactoperoxidase, and xanthine oxidase, collectively play vital roles in bolstering mammary gland immunity (Rainard and Riollet, 2006; Vlasova and Saif, 2021). For example, complement functions include opsonization, attraction to phagocytes, and the activation of intracellular killing of pathogens (Rainard, 2003). Lactoferrin is an iron-binding protein, with bacteriostatic properties, and is important in the mammary gland immunity against mastitis pathogens (Shimazaki and Kawai, 2017).

Cellular components such as macrophages and granulocytes also play important roles in the recognition and development of an effective inflammatory response to clear the infection (Rainard and Riollet, 2006). During inflammation, cytokines (e.g., Interleukin 1-beta, Interleukin 8, Interferon alpha, and Tumor Necrosis Factor alpha) and oxylipids play a role in facilitating the migration of white blood cells from the bloodstream (Oviedo-Boyso et al., 2007; Sordillo, 2018).

This process results in a rapid influx of neutrophils into mammary tissue. In uninflamed quarters, the predominant cell types are macrophages and lymphocytes; however, both the absolute number and relative abundance of neutrophils increase rapidly during inflammation (Halasa and Kirkeby, 2020). While it takes more time to establish itself compared to innate immunity upon exposure to microbes, adaptive immunity becomes crucial when pathogens can escape or withstand the innate defense system (Sordillo, 2018).

The components of adaptive immunity against mastitis involve the participation of T and B lymphocytes, leading to the subsequent production of immunoglobulins with the objective of neutralizing and eliminating pathogens (Sordillo, 2018). The labile balance between the mammary gland's immune system and mastitis-causing pathogens is disrupted in moments when the immune system's ability to fight against pathogens is affected, such as the transition from lactating to non-lactating status (i.e., dry-off) and the transition from dry to lactating period (Bradley and Green, 2004). During the transition period, period encompassing several weeks around parturition, multiple metabolic and physiological changes occur in preparation for the onset of a new lactation (Drackley, 1999). This stage is characterized by a rapid increase in the demands of nutrients from the mammary gland, and dry matter intake that is unable to provide the necessary nutrients, leading to a period of nutrient deficit (Caixeta and Omontese, 2021). To a certain extent, this occurs in all dairy cows, but if exacerbated, the mobilization of body adipose reserves could lead to the development of metabolic disorders, including hyperketonemia and fatty liver disease (Sundrum, 2015). These metabolic disorders disrupt the ability of the immune system to respond to pathogens, leading to a higher risk of acquiring mastitis (Ingvartsen and Moyes, 2013; Esposito et al., 2014). As an example, hyperketonemia

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compromises the immune system's ability to respond to antigens by inhibiting processes such as chemotaxis, oxidative burst, lymphocyte blastogenesis, and immunoglobulin production (Ingvartsen and Moyes, 2013).

Furthermore, the pro-inflammatory condition often observed in early lactation can result in a state of dysfunctional inflammation and oxidative stress, leading to an exaggerated response to infections that is, in most cases, ineffective (Sordillo and Mavangira, 2014). This situation is characterized by inadequate control of reactive oxygen species accumulation in the mammary gland, increasing the susceptibility of these animals to various health disorders and leading to tissue damage (Sordillo et al., 2009).

The transition period is especially challenging for nulliparous cows starting their first lactation, due to multiple management and physiological changes (De Vliegher et al., 2012). For instance, first-lactation animals are usually still growing at their first calving (Heinrichs et al., 2017). Furthermore, nulliparous cows are often grouped during the close-up period and at calving with older animals, which has a greater behavioral impact on h these cows compared to multiparous cows (Soonberg et al., 2021). They exhibit different behavior patterns during the transition period compared to multiparous cows (Neave et al., 2017). All the above could have an impact on the susceptibility to diseases during transition period including mastitis (Tucker et al., 2021).

2.3.3. Environment and other risk factors: Mastitis risk factors

The risk factors for mastitis development in conventionally managed first-lactation dairy cows have been widely investigated. For instance, it has been shown that the prevalence of

subclinical mastitis differs by season and herd location (Waage et al., 1998; De Vliegher et al., 2004b; Fox, 2009). Holstein heifers have been associated with a higher risk of mastitis in early lactation when compared to other breeds (Compton et al., 2007b; Bludau et al., 2014). Not surprisingly, heifers with a dirty udder around calving showed an increased risk of clinical mastitis (De Vliegher et al., 2004b; Compton et al., 2007b).

The presence of udder edema has been related to an increased risk of mastitis in early lactation heifers (Waage et al., 2001; Compton et al., 2007b). Udder edema is a noninfectious metabolic disorder in dairy cattle that results in the accumulation of liquid in the mammary gland tissue spaces (Okkema and Grandin, 2021). Multiple factors have been hypothesized as potential causes of udder edema including genetics, nutrition, oxidative stress, and physiological changes in freshening heifers (Okkema and Grandin, 2021). Milk leakage at calving has also been reported as a risk factor for mastitis in conventionally reared heifers (Waage et al., 1998). In a nested case-control study, the odds of milk leakage were 1.36 higher for heifers with clinical mastitis compared to controls (Waage et al., 1998).

Nutritional factors are also important risk factors for the development of mastitis. Selenium, vitamin A, vitamin E, copper, and zinc all played a role in the prevention of mastitis in heifers (Heinrichs et al., 2009). For example, vitamin E and selenium are crucial for inactivating reactive oxygen species, and their supplementation has been linked to a reduced risk of mastitis in some previous studies (Heinrichs et al., 2009). However, a recent meta-analysis showed no effect of vitamin E on SCC (Moghimi-Kandelousi et al., 2020). In another study that investigated supplementation with trace minerals, including selenium, copper, zinc, and manganese, it was found that the supplementation with these minerals led to lower SCC and reduced incidence of

subclinical mastitis, highlighting the vital role of these micronutrients in udder health (Machado et al., 2013).

Fly control has also been related to a lower risk of mastitis in conventionally managed heifers (De Vliegher et al., 2004b). This has been attributed to the fact that horn flies (*Haematobia irritans*) carry similar strains of *Staphylococcus aureus* to those identified in the mammary gland of dairy cows, suggesting that they could serve as biological vectors for the transmission of this microorganism (Gillespie et al., 1999; Anderson et al., 2012).

Lastly, heifers from farms with lower bulk tank SCC were less likely to calve with subclinical mastitis (De Vliegher et al., 2004b). This finding underscores the significance of adopting practices to maintain good herd-level udder health, thereby also diminishing the risk of heifers becoming infected prior to and at calving (De Vliegher et al., 2004b). This observation aligns with previous studies that demonstrated a positive association between the herd-level prevalence of *Staphylococcus aureus* IMI and the risk of IMI at the cow level, suggesting that the risk of transmission increases as the herd-level prevalence of IMI is higher (Dufour et al., 2012).

Compared to conventional dairy farms, information about the main risk factors for heifer mastitis on organic dairy farms is far more limited. A study performed on two large organic dairy farms showed that the presence of milk leakage in the first week of lactation, a high body condition score (>3.5) 6 weeks prior to calving, and udder edema were risk factors for the presence of mastitis at calving (Fernandes et al., 2022).

2.4. MASTITIS CONTROL PRACTICES

Despite significant advancements in animal management, genetic selection, and our understanding of mastitis epidemiology, mastitis remains a persistent issue on dairy farms (Ruegg, 2017). Traditionally, mastitis control plans have focused on the strategies outlined in the well-known 5-point plan (Neave et al., 1969). This plan included measures such as maintaining an adequate milking routine, ensuring the proper maintenance of milking machines, culling cows with chronic IMI, and administering antibiotic therapy for clinical mastitis during lactation and at dry-off for all quarters (Neave et al., 1969). This plan contributed to reducing the prevalence of contagious mastitis pathogens and bulk tank SCC in conventional dairy farms (Ruegg, 2017). Nonetheless, this plan appeared to show little effectiveness on the control of environmental mastitis.

More recently, the National Mastitis Council (**NMC**) created an extended 10 points control plan in which additional measures, such as maintaining cows in a clean, dry and comfortable environment, use of proper dry cow management and biosecurity measures to prevent the spread of contagious pathogens were included (NMC, 2016). Although the 10 point control plan includes various preventive strategies to prevent the acquisition of new IMI, antibiotics still play a crucial role to eliminate IMI (Krömker and Leimbach, 2017). This is not surprising, given the high efficacy of antibiotic treatment, especially at dry-off on the prevention and cure of IMI (Winder et al., 2019; McCubbin et al., 2022; Dziuba et al., 2023). This is especially important for gram-positive microorganisms, such as *Staphylococcus aureus* or *Streptococcus agalactiae,* that show high adaptation to the mammary gland (Rainard et al., 2018; Keane, 2019). These microorganisms lead to infections that can persist in the mammary gland

during the entire lactation or even across different lactations (Veh et al., 2015). In addition, IMI

caused by these microorganisms can be transmitted to other animals, magnifying the problem

and increasing the herd-level prevalence of a pathogen if control measures are not taken

(Barkema et al., 2009; Dufour et al., 2012). For this reason, antibiotic therapy or culling if

therapy is not successful, are important pillars for mastitis control on conventionally managed

dairy farms (Gussmann et al., 2019). A comparison between the measured included in the 5-

point and the 10-point control plans are presented in **Figure 2.1**.

Five-point control plan (Neave et al., 1969)	Ten-point control plan (NMC, 2016)
1. Good milking routine with post-milking teat disinfection 2. Maintenance of milk machine 3. Culling chronically infected cows 4. Treatment of all quarters at dry-off 5. Treatment of all cases of clinical mastitis during the lactation	1. Proper milking procedures 2. Proper maintenance and use of milking equipment 3. Maintenance of biosecurity for contagious pathogens and marketing of chronically infected cows 4. Effective dry cow management 5. Appropriate management of clinical mastitis during the lactation 6. Establishment of goals for udder health 7. Maintenance of a clean, dry, comfortable environment 8. Good record keeping 9. Regular monitoring of udder health status 10. Periodic review of mastitis control program

Figure 2.1. Points included in the 5-point mastitis control plan (1969) and updated National
Mastitis Council (NMC) recommended 10-point mastitis control plan (2016).

2.5. ANTIMICROBIAL STEWARDSHIP ON DAIRY FARMS

In the last few years, there has been increasing concern about the use of antimicrobials on

dairy production farms related to their potential negative impacts on animal and human health,

through the potential for selection of antimicrobial resistance (Krömker and Leimbach, 2017).

For this reason, many farms are adopting strategies to improve antimicrobial stewardship such as

the selective treatment of clinical mastitis cases or selective dry cow therapy (McCubbin et al., 2022; de Jong et al., 2023). Research has shown that in well-managed herds these strategies can be implemented successfully, reducing antimicrobial use, without negatively impacting udder health (McCubbin et al., 2022; de Jong et al., 2023).

2.6. ORGANIC DAIRY MANAGEMENT AND UDDER HEALTH

A particular case in which restrictions are placed on the use of antimicrobials are organic dairy farms (NMC, 2019). Although they represent a small proportion (3.6%) of dairy production in the United States (USDA, 2022), the number of organic dairy farms is surging swiftly, with a five-fold increase over the last two decades (Dimitri and Nehring, 2022). Organic dairy farms are under the regulation of the United States Department of Agriculture as implemented in the National Organic Program. The National Organic Program created a set of norms and regulations that upholds the requirements for producing organically produced agricultural products in the United States (USDA, 2023). According to the regulations of this program, organic dairy farms are only allowed to use non-synthetic (i.e., natural) substances for dairy production. Non-synthetic substances are defined as those produced from a natural source (e.g., mineral, plant, or animal matter) that have not undergone chemical changes not related to natural processes. In addition to those defined as non-synthetic substances, a few synthetic substances are allowed in organic dairy production in response to a National Organic Standards Board recommendation. These exceptions currently include dietary supplements such as vitamins, amino acids, as well as certain parasiticides, drugs, analgesics, and vaccines. In addition, there are strict regulations about the diet of organically raised dairy cows, requiring that all the feed offered to dairy cows in organic production systems must come from organically certified sources. Furthermore, cows are

required to have at least 30% of their dry matter intake from pasture, when possible (i.e., during the grazing season), to comply with organic regulations. Related to udder health, the most important restrictions under the organic banner in the United States is the prohibition of the use of internal teat sealants and the restrictions on the use of antibiotics in dairy cows (NMC, 2019).

Organic dairy farms cannot use antibiotics to control mastitis, as well as other infectious diseases. However, antibiotics must be used to avoid animal suffering when organic approved methods are ineffective. In this situation, the animal treated with antibiotics cannot maintain its organic status and must leave the herd (USDA, 2023). This represents an important challenge, due to the important role that antibiotics play in mastitis control plans (McCubbin et al., 2022; de Jong et al., 2023). Likewise, internal teat sealants are an important preventive measure on conventional dairies (Pearce et al., 2023). This is important given the high risk of the acquisition of IMI during the dry period (Bradley and Green, 2004) and is particularly crucial in cases in which dry cow therapy is not administered to dairy cows (Kabera et al., 2021). A recent meta-analysis showed that internal teat sealants and dry cow therapy antibiotics can be used to reduce the prevalence of postpartum mastitis in transitioning heifers (Naqvi et al., 2018). The challenges outlined above represent significant issues for organic dairy farms, which need the implementation of alternative prevention strategies to maintain udder health.

An important research question is whether these restrictions around the use of antibiotics or teat sealants could impair the udder health in organic dairy cows. Some studies have reported that organic dairy farms have an increased prevalence of *Staphylococcus aureus* compared to conventional dairy farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). Additionally, it has been noted that organic dairy farms have a higher bulk tank SCC than conventional dairy farms

(Zwald et al., 2004; Levison et al., 2016). However,there are some contradictory findings (Vaarst et al., 2001; Valle et al., 2007; Stiglbauer et al., 2013). Interestingly, previous reports from Europe (Hamilton et al., 2006; Valle et al., 2007) and North America (Richert et al., 2013) report that clinical mastitis incidence in conventional dairy farms in lower compared to organic dairies. Nevertheless, comparisons of the rates of clinical mastitis in conventional and organic dairies need to be interpreted with caution since the detection and diagnosis of clinical mastitis are affected by the management system (Ruegg, 2009). Therefore, it is possible that organic dairy farms may not be identifying or may be underreporting clinical mastitis cases.

Despite the differences in clinical mastitis and bulk tank SCC, no differences in the prevalence of subclinical mastitis (Hardeng and Edge, 2001) or cow-level SCC (Mullen et al., 2013) were observed comparing organic and conventional dairies. It is important to highlight, however, that organic dairy farms may have risk factors unrelated to organic practices that can impact the risk of mastitis (Ruegg, 2009). For instance, a previous observational study that collected data from 192 organic dairy farms, showed that, in comparison to conventional dairies, organic dairy farms tend to be smaller, were less likely to have regular veterinary visits, less likely to use vaccines for disease control, and were less likely to maintain records of treatments administered to animals or be enrolled in dairy herd improvement association testing services (Stiglbauer et al., 2013).

Considering all these observations together, in addition to the restrictions imposed by organic production norms and regulations, it becomes apparent that organic dairy farms may be at a higher risk of mastitis compared to conventional dairy farms. Nonetheless, the amount of information about mastitis epidemiology and udder health on organic dairy farms is scarce.

Particularly, while prior studies have primarily focused on management and udder health at the herd level, the prevalence and distribution of mastitis pathogens at the individual cow level on organic dairies are not well understood.

2.7. MASTITIS IN FIRST-LACTATION ORGANIC DAIRY COWS

Disease prevention is especially important in first-lactation dairy cows. Organic dairy farms face additional challenges because replacement heifers need to be raised organically or they need to be from organically managed farms (USDA, 2023). Hence it is not surprising that the heifer rearing costs are higher than those for conventional dairy farms (McBride and Greene, 2007). Thus, the longevity of organically reared cows can be especially important for the sustainability of organic dairy operations (Horn et al., 2012; Lopez-Villalobos et al., 2018). Prior reports have shown that early lactation primiparous cows have a higher incidence of clinical mastitis compared to multiparous cows (De Vliegher et al., 2012). The presence of clinical mastitis in early lactation heifers has been associated with an increased risk of clinical mastitis over their lifetime (Hertl et al., 2018). In addition, the presence of subclinical (i.e., high SCC) or clinical mastitis has been associated with an increased culling rate (Hertl et al., 2018; Fernandes et al., 2021). For instance, it was reported that organic cows with high SCC in the first month postpartum had twice the hazards of herd removal compared to those without high SCC (Fernandes et al., 2021). In addition, the hazards of herd removal increased 1.34 times for each additional case of clinical mastitis in the first 100 days in milk (Hertl et al., 2018).

The prevalence of major mastitis pathogens (i.e., *Staphylococcus aureus* and *Streptococcus* spp.) appears to be lower in first-lactation dairy cows. Despite this, some reports have shown a high prevalence of Staphylococcus aureus on conventional dairies even before

calving (De Vliegher et al., 2012). As mentioned previously, this could be the case for organically reared heifers in which the use of antibiotics for herd-level management of mastitis is prohibited (NMC, 2019), and there is an observed higher herd-level prevalence of *Staphylococcus aureus* (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013).

All the above has increased awareness about the need for research on mastitis in this subgroup of animals, to look for potential strategies to improve udder health and minimize the negative impacts of mastitis on heifers' performance. However, many questions remain unanswered. For instance, what is the impact of restrictions on organic dairy farms on the udder health of first-lactation dairy cows? What is the distribution of NASM species on organically raised heifers, which are commonly found mastitis pathogens in conventional dairy heifers? Is there a difference in the relationship between the presence of IMI and udder health on organic dairy farms, compared to conventional dairy farms? Lastly, are there any promising evidence-based strategies for mastitis therapy that could serve as alternatives to antibiotics?

2.8. APPROACHES FOR MASTITIS TREATMENT ON ORGANIC DAIRY FARMS

Multiple studies have investigated the possibility of developing alternative products to antimicrobials, but with limited success. Homeopathy is a type of therapy that has gained popularity in veterinary medicine; however, most studies have failed to demonstrate an effect compared to controls (Mathie and Clausen, 2015). For instance, randomized controlled trials showed no evidence of an effect on clinical mastitis cure for cows administered homeopathy compared to a placebo (Hektoen et al., 2004; Keller and Sundrum, 2018). Despite lacking evidence supporting homeopathy, these products are widely used for mastitis control on organic dairy farms (Ruegg, 2009).

Phytotherapeutics have been investigated as a potential way of treating mastitis in dairy farms (Lopes et al., 2020). While *in vitro* studies demonstrated the inhibitory activity of plant-based products such as trans-cinnamaldehyde, caprylic acid, and monocaprylin on mastitis pathogens (Nair et al., 2005; Ananda Baskaran et al., 2009), *in vivo* studies have failed to show their effectiveness in treating clinical mastitis (Mullen et al., 2018; Caneschi et al., 2023).

Another potential alternative treatment approach that could be used by organic farms are phage-based products (Dias et al., 2013; Kanwar et al., 2023). These products contain bacteriophages, viruses that infect, replicate, and destroy specific bacterial microorganisms (O'Sullivan et al., 2020). Phages-based products have been successfully used in murine models to treat *Staphylococcus aureus* mastitis (Geng et al., 2020; Ngassam-Tchamba et al., 2020), representing a potential alternative to treat bovine mastitis. However, this utilization of phage therapy to treat mastitis requires more investigation.

Lastly, one area of great interest in mastitis treatment relies on the use of bacteriocins, antimicrobial peptides (**AMPs**) produced by bacteria (de Freire Bastos et al., 2020; Newstead et al., 2020). These have the advantages of having a narrower spectrum of action compared to antibiotics, and show different mechanisms of action making them a potential alternative for the treatment of antimicrobial resistant strains (de Freire Bastos et al., 2020; Newstead et al., 2020). Many of these bacteriocins have been suggested as a potential alternative for the treatment of mastitis (Nascimento et al., 2005; Braem et al., 2014; Carson et al., 2017). One of the most promising candidates is nisin, an AMP produced by *Lactococcus lactis* (Cao et al., 2007; Wu et al., 2020). In two prior randomized controlled trials, treatment with nisin showed a clinical cure rate comparable to that of an intramammary antibiotic (Cao et al., 2007), and a higher clinical

cure rate compared to that of quarters with no treatment (Wu et al., 2020). It is important to note that these studies were conducted on a small scale at a single farm. Thus, further investigation is needed to assess the potential use of this product for treating mastitis in larger populations of dairy cows and farms.

Although many studies have explored different approaches to treat mastitis with some promising *in vitro* results, most of the studies have provided only limited or no evidence of effectiveness *in vivo*. Therefore, more research is needed to identify effective non-antibiotic alternative methods to treat mastitis.

2.9. MICROBIOME-BASED PRODUCTS AS AN ALTERNATIVE FOR MASTITIS CONTROL

In recent years, the cost of DNA sequencing has significantly decreased, resulting in an increased number of studies using metagenomic approaches to investigate the role of commensal bacteria in animal health (Peixoto et al., 2021). The investigation of the udder microbiome has given us an insight into the complex community of microorganisms that are present in the healthy mammary gland (Derakhshani et al., 2018; Ruegg, 2022a). This leads to the question of whether some of these microorganisms could be used to control mastitis. Hence, one could hypothesize that these microorganisms could modulate the composition of the teat skin and milk microbiota or the presence of pathogenic microorganisms in the mammary gland (Rainard and Foucras, 2018). Unfortunately, microbiome studies show inconsistent results regarding which microorganisms have a protective role on the mammary gland, hence, practical applications of the microbiome to control mastitis are still not available (Derakhshani et al., 2018; Ruegg, 2022a).

2.10. NON-AUREUS STAPHYLOCOCCI AND CLOSELY RELATED MAMMALOLIICOCCAL SPECIES AND THEIR POTENTIAL TO CONTROL MASTITIS ON ORGANIC DAIRY FARMS

2.10.1. Role of NASM in udder health

Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species have emerged as a potential candidate for the development of products to treat and prevent mastitis on organic dairy farms. This is based on their high prevalence in the mammary gland (De Buck et al., 2021), evidence of *in vivo* protective activity against major mastitis pathogens in experimental studies (Reyher et al., 2012), and reports that showed lower clinical mastitis, and higher milk production in cows with NASM IMI (Compton et al., 2007a; Piepers et al., 2010, 2013).

In recent years, multiple studies have shown that NASM possesses *in vitro* antimicrobial activity (De Vliegher et al., 2004c; Braem et al., 2014; Carson et al., 2017). A Belgian study identified antimicrobial activity in 38 out of 254 (15.0%) NASM isolates (Braem et al., 2014). Similarly, a Canadian study reported that 40 out of 441 (9.1%) NASM isolates displayed the ability to inhibit *Staphylococcus aureus in vitro* (Carson et al., 2017). More recently, agar dilution methods have been employed to test multiple concentrations of NASM against mastitis pathogens *in vitro*. This methodology enabled the identification of the minimum concentration required to stop the growth of *Staphylococcus aureus*, *Streptococcus uberis*, and *Escherichia coli* (Toledo-Silva et al., 2022). In this study, it was shown that the inhibitory activity of NASM against *Staphylococus aureus*, *Streptococcus uberis,* and *Escherichia coli* was variable, a finding not evident using the previously used semi-quantitative method (Toledo-Silva et al., 2022).

The *in vitro* antimicrobial activity present in some NASM strains has been attributed to the production of AMPs (Nakatsuji et al., 2017). These short-chain peptides have shown antibacterial properties and could be targeted for developing of alternative methods to combat mastitis (Nascimento et al., 2005; Braem et al., 2014; Carson et al., 2017). Bacteriocins from gram-positive bacteria are commonly classified into 3 categories (Rea et al., 2011). Class I bacteriocins include post-translationally modified bacteriocins, such as lantibiotics, labyrinthopeptins, and sactibiotics (Rea et al., 2011). Class II comprises unmodified bacteriocins originating from a diverse range of peptides, including pediocin-like bacteriocins, two-peptide unmodified bacteriocins, circular bacteriocins, and unmodified, linear, non-pediocin-like bacteriocins (Rea et al., 2011). Lastly, bacteriolysins, also known as Class III bacteriocins, are characterized as large, heat-labile bacteriocins (Rea et al., 2011). Many of these bacteriocins have been identified in bovine NASM (Nascimento et al., 2005; Braem et al., 2014; Carson et al., 2017).

In addition to bacteriocins, auto-induced peptides (**AIPs**) are substances involved in quorum sensing through the activation of the accessory gene regulation (**agr**), that are important for *Staphylococcus aureus* expression of virulence genes and pathogenesis (Le and Otto, 2015; Wang and Muir, 2016). Auto-induced peptides are also produced by NASM (Williams et al., 2023), and can inhibit the agr system in *Staphylococcus aureus*, disrupting its virulence potential (Canovas et al., 2016; Toledo-Silva et al., 2021). Hence, the development of solutions containing AIPs has been proposed as potential therapeutic alternative to control *Staphylococcus aureus* infections (Gray et al., 2013; Tan et al., 2018).

2.10.2. Characterization of NASM using whole genome sequencing

Considering the divergent epidemiological behavior across and within NASM species, it is of great interest to characterize the NASM genome (De Buck et al., 2021). The use of next generation sequencing allows us to subject NASM genomes to whole genome sequencing for only a fraction of what this would cost in the past (Christensen et al., 2015). Nonetheless, only a few studies have investigated the entire genome of NASM utilizing whole genome sequencing (De Buck et al., 2021).

A fundamental area of interest could be related to the phylogeny of NASM species. This exploration seeks to elucidate evolutionary changes that could explain common epidemiological traits. A previous study that characterized 441 strains from 25 different NASM species revealed that these species formed 5 distinct clades. This finding provide insights into shared biological characteristics across different NASM species, including their virulence, environmental niche, and geographical distribution (Naushad et al., 2016). However, further studies are warranted to explore their distribution, especially for species with limited sample sizes.

Furthermore, due to the significant variations in virulence potential among different NASM species and the contradictory findings regarding their impact on udder health, milk production, or their ability to persist in the mammary gland (De Buck et al., 2021), it is of great interest to investigate the genetic determinants of NASM virulence. Traditional classifications of NASM as commensals gave *Staphylococcus aureus* the spotlight. However, in recent years, an increasing amount of studies have investigated the virulence potential of NASM and their effects on both human and animal health (Taponen and Pyörälä, 2009; Heilmann et al., 2019; França et al., 2021).

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Staphylococcus spp. have a variety of virulence factors (**VFs**) that could explain their success in mammary gland colonization and the detrimental effects of some species on udder health (Rainard et al., 2018; Campos et al., 2022). During mammary gland invasion, microorganisms enter the mammary gland through the teat canal. After this, colonization of the mammary gland is facilitated by the presence of cellular components present on the microbial surface commonly referred to as adhesins (Campos et al., 2022). These promote adhesion and invasion of the mammary gland by recognition of cell components present in mammary gland cells and the creation of bridges between bacterial and epithelial cells (Campos et al., 2022). In *Staphylococcus aureus*, fibronectin-binding proteins (**fnb**) play a significant role in adhesion to the mammary gland (Campos et al., 2022). Notably, *Staphylococcus aureus* exhibited a high proportion of isolates with the presence of genes encoding fnb (41/45; 91%), as well as other VFs, including autolysin (45/45; 100%) and intracellular adhesion proteins (**ica**) (Fergestad et al., 2021).

Autolysins are cell wall-lytic enzymes with adhesive properties that mediate binding to host proteins and play important roles in host cell adhesion (Otto, 2004). The presence of these genes is frequently observed among NASM species. This is demonstrated by a prior study that reported a prevalence of 68% in 25 species (Naushad et al., 2019), while another study found a prevalence of 35.3% in 17 species (Fergestad et al., 2021). Additionally, genes associated with biofilm formation, such as icaC (17/25; 68%), icaA (8/25; 32%), icaB (7/25; 28%), icaD (8/25; 32%), and icaR (7/25; 28%), appear to be highly prevalent in NASM species (Naushad et al., 2019). Other VFs such as fnbB showed a low prevalence and were only identified in

Staphylococcus agnetis, *Staphylococcus hyicus*, *Staphylococcus chromogenes* (Naushad et al., 2019) or in another study in none of the NASM species (Fergestad et al., 2021).

Exoenzymes are crucial for the invasion and colonization of *Staphylococcus* spp. (Tam and Torres, 2019). Previous studies have revealed that the genome of *Staphylococcus aureus* contains genes encoding a wide array of VFs, including adenosine synthase, aureolysins, cysteine proteases, hyaluronate lipases, serine proteases, staphylokinase, thermonuclease, and von Willebrand factor-binding proteins. Interestingly, nearly all of these VFs can be identified in bovine *Staphylococcus aureus* isolates (Fergestad et al., 2021). In contrast, among NASM, the most prevalent exoenzymes are thermonucleases, lipases, and aureolysins, while other exoenzyme genes exhibit a lower but divergent prevalence across different species (Naushad et al., 2019; Fergestad et al., 2021).

Virulence genes associated with host immune evasion play an important role in *Staphylococcus aureus* pathogenesis (Campos et al., 2022). These include genes involved in capsular synthesis (**cap**), chemotaxis inhibition (**chp**), complement inhibition (**scn**), Staphylococcal protein A (**spa**), and staphylococcal binder of immunoglobulin (**sbi**). These VFs showed a high prevalence in *Staphylococcus aureus* bovine isolates, with nearly all of these genes found in their genomes, except for chp (Fergestad et al., 2021). Conversely, NASM showed a high but varying prevalence of different cap genes in which *Staphylococcus agnetis*, *Staphylococcus hyicus*, *Staphylococcus chromogenes, Staphylococcus haemolyticus, Staphylococcus pasteuri* and *Staphylococcus areletteae* showed a high prevalence in NASM genomes in 2 different observational studies (Naushad et al., 2019; Fergestad et al., 2021).

Iron uptake and metabolism genes are also essential for the survival of *Staphylococcus* spp. (Hammer and Skaar, 2011; França et al., 2021). Previous studies have identified multiple genes related to iron uptake and metabolism, including iron-regulated surface determinants, ABC transporters, and Staphyloferrins synthesis, in both *Staphylococcus aureus* and NASM bovine strains (Naushad et al., 2019; Fergestad et al., 2021).

Staphylococcus spp. possess multiple genes that encode for toxins that play a role in disease pathogenesis (Campos et al., 2022). These toxins include hemolysins, leukocidins, toxic shock syndrome toxins, exfoliative toxins, and alpha/beta modulins. The majority of these toxin genes were highly prevalent in *Staphylococcus aureus* but were not identified in NASM species, with a few exceptions (Naushad et al., 2019; Fergestad et al., 2021). For example, beta-hemolysins, exfoliative toxin type C, and various phenol-soluble modulins were found in NASM, but the results were inconsistent across studies (Naushad et al., 2019; Fergestad et al., 2021).

Lastly, with the rise in antimicrobial resistance among human pathogens and the potential implications for both human and animal health, there has been an increasing level of concern surrounding this issue (Roca et al., 2015; McEwen and Collignon, 2018). Given the commensal nature and high prevalence of NASM, it has been plausible that these microorganisms could harbor genes that could be transferred to other pathogenic bacteria, such as *Staphylococcus aureus*, and conversely, that genes from pathogenic bacteria could be transferred to NASM (De Buck et al., 2021). Indeed, it has been shown that similar plasmids were identified in strains of different staphylococcal species, suggesting that plasmids can move across bacterial species within this genus horizontally (Fišarová et al., 2019, 2021).

In prior studies, it has been shown that NASM antimicrobial resistance was related to the administration of systemic but not intramammary antibiotics (Nobrega et al., 2018a; Stevens et al., 2018). In a Swedish investigation (Waller et al., 2011), resistance to more than one antimicrobial agent was found in 9% and 7% of subclinical and clinical mastitis isolates, respectively. Furthermore, it was discovered that beta-lactamase-producing isolates were more prevalent in cows with subclinical mastitis than in cows with clinical mastitis, especially when comparing *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* to other species (Waller et al., 2011). In addition, the presence of antimicrobial resistance genes (**ARGs**) has previously been linked to the development of phenotypic resistance in a Canadian study (Nobrega et al., 2018b). In this investigation, an important variation in the prevalence of ARGs was observed across different NASM species. The most prevalent ARGs were those that encoded tet38 efflux pumps (30%), macrolide phosphotransferase C (10%), and the beta-lactamase gene Z (6%) (Nobrega et al., 2018b).

2.11. CONCLUSIONS

Studies have provided evidence to suggest that udder health on organic dairy farms may be compromised due to the restrictions imposed by organic management. However, there is a limited body of knowledge available on this subject. Furthermore, prior research has shown limited success in developing mastitis treatment products for use in organic dairy farming, with no commercial alternatives to antibiotics currently available. Therefore, further investigation in this area is crucial to explore potential methods for mastitis control without the use of antimicrobials. Lastly, only a few studies have conducted whole genome sequencing on NASM

isolates, and additional research is needed to gain a better understanding of their antimicrobial capabilities, VFs, and potential for antimicrobial resistance.

CHAPTER 3: INVESTIGATION OF INTRAMAMMARY INFECTIONS IN PRIMIPAROUS COWS DURING EARLY LACTATION ON ORGANIC DAIRY FARMS

3.1 SUMMARY

Previous studies have shown that organically-raised dairy cows have an increased prevalence of *Staphylococcus aureus* compared to conventionally-raised dairy cows. However, little information exists about the dynamics of intramammary infections (**IMI**) in primiparous cows during early lactation on organic dairy farms. The objective of this study was to describe the IMI dynamics of primiparous cows on certified organic farms during early lactation. This longitudinal study enrolled 503 primiparous cows from 5 organic dairy farms from February 2019 to January 2020. Quarter-level milk samples were collected aseptically on a weekly basis during the first 5 weeks of lactation. Samples were pooled by cow and time point into composite samples inside a sterilized laminar hood and submitted for microbiological culture. For each of the different microorganisms identified, we estimated the prevalence in each postpartum sample, period prevalence (**PP**), cumulative incidence (**CmI**) and persistence of IMI. Logistic regression models were used to investigate whether the prevalence of IMI differed by farm or sampling time points and whether IMI persistence differed between detected microorganisms. Our findings revealed a high cow-level period prevalence of *Staphylococcus aureus* (PP = 18.9%), non-*aureus Staphylococcus* and closely related *Mammaliicoccal* species (PP = 52.1%), and *Streptococcus* spp. and *Streptococcus*-like organisms (PP = 32.1%) during the first 35 days in milk within the study population. The prevalence of these microorganisms varied significantly between farms. *Staphylococcus aureus* and *Staphylococcus chromogenes* exhibited significantly higher IMI persistence compared to other detected bacterial taxa, confirming the divergent epidemiological behavior in terms of IMI chronicity across different microorganisms. This study improves our understanding of the epidemiology of mastitis-causing pathogens in organically-raised

primiparous cows, which can be used to tailor mastitis control plans for this unique yet growing subpopulation of dairy cows.

3.2. INTRODUCTION

Recently, there has been increased demand for organic dairy products from consumers (Greene and McBride, 2015; Gundala and Singh, 2021). Although organic dairy production represents a small proportion of the overall milk sales in the U.S., demand continues to grow annually (USDA, 2021). U.S. farms that produce organic-certified milk operate under different regulations than conventional dairy farms, including restrictions on the use of antimicrobials. These additional restrictions can create challenges in maintaining udder health in organically-raised cows compared to conventionally-managed cows (Ruegg, 2009; NMC, 2019).

Mastitis is the main reason for administration of antimicrobials to dairy cows in conventional farms in the U.S. (USDA, 2016). Indeed, the use of intramammary antibiotics is a key component of many mastitis control plans on conventional farms. This is especially true at dry-off (Halasa et al., 2009a), and for the treatment of cases caused by microorganisms with a contagious epidemiology and high adaptation to the mammary gland (Barlow, 2011). The fact that antibiotics are a key component of mastitis control and treatment on conventional farms could indicate that organically raised cows may be at increased risk of mastitis because of restrictions on antibiotic use on organic farms (Ruegg, 2009; NMC, 2019).

While the epidemiology of mastitis on organic farms has not been extensively studied, available reports suggest they have an elevated prevalence of *Staphylococcus aureus* compared to conventional dairy farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). Bulk somatic

cell count (**SCC**) has also been reported to be higher on organic compared to conventional farms (Zwald et al., 2004; Levison et al., 2016), though conflicting reports do exist (Vaarst et al., 2001; Valle et al., 2007; Stiglbauer et al., 2013). In contrast, the incidence of clinical mastitis on organic dairy farms has been reported to be lower than conventional farms (Hamilton et al., 2006; Valle et al., 2007; Richert et al., 2013). Additionally, no differences have been found in the incidence of subclinical mastitis (Hardeng and Edge, 2001) or individual SCC (Mullen et al., 2013) on organic versus conventional farms. Such reports suggest that there may be differences in mastitis epidemiology between conventional and organic dairy farms.

First-lactation cows represent a particular concern in terms of mastitis epidemiology, as they constitute a significant investment during the rearing phase and serve as a replacement for older cows that leave the herd (Boulton et al., 2017). These costs are even higher for organic dairy farms, especially those that are transitioning from organic to conventional dairy farming, because the available pool of organically raised calves is much more constrained and raising an organic-certified cows has additional costs. Studies in conventional herds consistently report that primiparous cows have a greater incidence of clinical mastitis in early lactation compared to multiparous cows (De Vliegher et al., 2012). In addition, primiparous cows with high SCC in early lactation have an increased SCC during the entire first lactation (De Vliegher et al., 2004a); in organically managed primiparous cows, this high SCC may also carry over into subsequent lactations (Fernandes et al., 2021).

It should also be noted that the management practices and herd characteristics of organic dairies vary dramatically across the U.S. and compared to conventionally managed farms (Stiglbauer et al., 2013). The U.S. has experienced the growth of relatively large organic dairy

farms, associated with increased profitability as herd size increases (Walsh et al., 2020). These operations typically employ different management strategies than the smaller organic dairy farms that characterized the U.S. organic dairy industry until recently (Stiglbauer et al., 2013). Observations regarding mastitis in organic dairy herds have typically originated from smaller herds (Cicconi-Hogan et al., 2013; Levison et al., 2016) and may not extrapolate well to larger herds.

Given the increasing number of organic farms in the United States, there is a growing need to better understand the dynamics of mastitis in these farms, particularly those with large herd size. Specifically, very little is known about the prevalence and characteristics of intramammary infections (**IMI**) in early lactation primiparous dairy cows raised under organic conditions. Therefore, the overall goal of this observational study was to describe the IMI dynamics of primiparous cows on certified organic farms during early lactation. Specific objectives were to estimate the prevalence and incidence of IMI with microorganisms of interest for udder health; and to describe their persistence in the mammary gland during the first 5 weeks of lactation.

3.3. MATERIALS AND METHODS

The Strengthening the Reporting of Observational Studies in Epidemiology - Veterinary (**STROBE-Vet**) Statement guidelines were followed for the preparation of this manuscript (Sargeant et al., 2016). All study activities were approved by the University of Minnesota Institutional Animal Care and Use Committee (**IACUC**) (Protocol Number: 1807: 36109A), Colorado State University IACUC (Protocol number: 1442) and Texas Tech University IACUC (Protocol number: 18068-10).

3.3.1. Herd inclusion criteria

Only USDA organic certified dairy farms were enrolled in this study. Farms were selected from different United States regions and herd sizes. Enrollment was based on willingness to participate in the study, availability of electronic farm records and proximity to the Universities involved in the study. For this longitudinal observational study, we enrolled 503 primiparous cows from 5 organic dairy farms (Farm A=162, Farm B=122, Farm C=130, Farm D=23, Farm E=66). The five dairy farms included in this study were selected based on their willingness to participate in the study. A total of 20 organic dairy farms were contacted by the beginning of the study. All cows that calved for the first time between February 2019 and January 2020 were eligible for enrollment. This study is part of a larger research initiative to investigate potential associations between the udder microbiome and udder health (Dean et al., 2021). For this purpose, cows were enrolled 8 weeks before calving and followed up during the first 5 weeks of lactation. In this study, we focus on the milk samples collected at calving and during lactation.

3.3.2. Milk sampling

Milk samples were collected by the research team. All research technicians collecting milk samples at different farms were trained to collected aseptic milk samples prior to the beginning of the study. Aseptic quarter milk samples were collected on a weekly basis during the first 35 days in milk (**DIM**) following procedures described by the National Mastitis Council (NMC, 2017). Briefly, 3 to 4 streams of milk were discarded after pre-dipping. Teat-ends were then thoroughly scrubbed with gauze squares soaked in 70% ethanol. Wearing clean gloves, quarter-level milk samples were collected in separate tubes. All samples were collected prior to

morning milking. Samples were stored on ice immediately upon collection and frozen at -20 °C within 4 hours of collection.

3.3.3. Milk pooling

Before submission of samples for milk culture, all available quarter samples from each cow were pooled into a single sample inside a sterilized laminar hood. Briefly, quarter samples were thawed overnight at 4 °C. After homogenization, 2 mL of milk was extracted from each quarter samples and dispensed into a single sterile plastic vial. The resultant composite milk samples were then submitted to the Laboratory for Udder Health at the University of Minnesota for milk culture.

3.3.4. Milk culture

Using a cotton swab, milk (approximately 100 μL) was plated onto Columbia CNA agar with 5% sheep blood and MacConkey agar. Agar plates were incubated in aerobic conditions at 37°C for 42 to 48 h. Subsequently, plates were visually examined by a trained technician, and the identity of representative colonies was further investigated. Milk samples were defined as contaminated when more than 3 distinct isolates were identified across the two utilized plates (Dean et al., 2022). Taxonomic assignment of isolates was accomplished in non-contaminated milk samples using a Matrix-assisted laser desorption/ionization-time of flight (**MALDI-TOF**) mass spectrometer (**MS**) (MALDI Microflex LT Biotyper, Bruker Daltonics Inc.) as previously described (Jahan et al., 2021). Briefly the peak profiles of each of the isolates were compared to a reference spectra Biotyper reference library (Microflex version 7854; last updated on 02/19/2019, Bruker Daltonics Inc.). Following manufacturer's recommendations, confidence

scores were used in the following way: >2.0: species-level diagnosis; 1.8 to 2: genus-level

diagnosis and <1.8: MALDI-TOF diagnosis not recorded and traditional identification methods

used. An IMI was defined as a composite sample containing one or more colony forming units

(i.e., 10 colony forming units (**CFU**)/mL) of any cultured isolate. Microorganisms were grouped

into different taxonomic groups: *Staphylococcus aureus*; non-*aureus Staphylococcus* spp. and

the closely related *Mammaliicoccal* species (**NASM**); *Streptococcus*-like organisms (**SLO**,

comprising *Enterococcus* spp., *Aerococcus* spp. *Lactococcus* spp. and *Micrococcus* spp.);

Streptococcus spp. and *Streptococcus*-like organisms (**SSLO**, which include members from the

Streptococcus genus as well as the SLO); gram negative bacteria (i.e.,, *Escherichia* spp.,

Klebsiella spp., *Enterobacter* spp., *Pseudomonas* spp., *Enterobacter* spp., *Pantoea* spp.) and

"others" (i.e., microorganisms that did not belong to any of the previous groups). Milk samples

with no calving date information, contaminated milk samples, milk samples from cows with no

sample in the first 14 DIM, and milk samples corresponding to a 6th postpartum sample and/or

collected after 35 DIM were excluded from the analysis (**Figure 3.1**).

3.3.5 Definition of postpartum sample

Given complexities of farm management and sampling logistics, the interval between

sample collections was not always perfectly consistent for every enrolled animal, i.e., the

sample-to-sample interval was not always exactly 7 days. Consequently, for some cows, two

samples may have been collected within 1 week of each other (i.e., 6 days apart). Additionally,

some animals could not be sampled during the first postpartum week, and/or the milk samples in

that period were contaminated. Given these considerations and our desire to understand IMI

persistence, we decided to use the order in which the postpartum samples were collected as a

surrogate for postpartum week (i.e., the first sample collected was considered "postpartum sample 1", the second sample "postpartum sample 2", etc…). To understand the distribution of DIM relative to sample collections, see **Supplementary Figure 3.1** (https://doi.org/10.6084/m9.figshare.24194691.v1). A total of 424 cows were included in the analysis, from which 20 out of 424 (4.7%) had the results available from 1 sample only, 31 out of 424 (7.3%) from 2 samples and 373 out of 424 had the results available from 3 or more samples (88.0%).

3.3.6. Statistical analysis

Statistical analysis was performed using R (https://www.r-project.org/; version 4.1.2). The statistical code used for this study, including all relevant outputs, can be found online (https://fepenamosca.github.io/IMI-organic-dairies.github.io/). Prior to the start of analysis, electronic and paper data were manually compared to correct any errors in sample labeling or collected records (e.g., incorrect animal tags, farm names, date of sample collection and calving dates). When inconsistencies were present in the electronic records, the errors were corrected based on the cow-ID, sampling date recorded on milk vials, and sampling paper records (i.e., gold standard records).

Estimation of prevalence, cumulative incidence, and persistence of intramammary infections. Period prevalence (**PP**) of IMI was calculated as the proportion of animals that had an IMI during the postpartum period. Additionally, prevalence of IMI at calving was defined as animals that had an IMI in the first postpartum sample. The proportion of IMI by each microorganism that was already present in the first postpartum sample was reported. Cumulative incidence (**CmI**) was defined as the proportion of animals at risk that acquired a new IMI by a particular

microbe at any given sampling following the first sampling (e.g., sample 2, 3, 4 or 5), but without an IMI by that microorganism in the first postpartum sample. Postpartum sample prevalence was calculated as the proportion of animals that had an IMI at a specific postpartum sample. Persistence of IMI was calculated by summing the number of postpartum samples in which cows harbored the same microorganism. Persistent-IMI was defined as harboring the same microorganism for 2 or more samples after calving, regardless of whether the IMI occurred in consecutive samples or not. Only cows with at least two samples collected during the follow-up period (n=404) were included in the analysis of persistence, CmI, and the proportion of IMI identified in the first postpartum sample.

Associations between farm, postpartum sample and intramammary infections. To investigate differences in IMI prevalence across postpartum samples and farms, we utilized multivariable modeling. Association between postpartum sample (explanatory variable) and presence of IMI for each bacterial group (dependent variable) was assessed using mixed logistic regression as implemented in 'lme4' (Bates et al., 2015). Farm-ID and cow-ID were included as fixed and random effects in the models, respectively, to account for the non-independence of observations within each sampling unit. Association between farm (explanatory variable) and PP of IMI (dependent variable) was investigated using logistic regression.

Association between bacterial group and persistence of intramammary infections. The association between bacterial group and persistence in the mammary gland was assessed using mixed logistic regression as implemented in 'lme4' (Bates et al., 2015). Potential confounding due to different number of samples available for each cow was accounted for by including this variable as a covariate in the model. Since each animal could have multiple IMI during the

postpartum period, cow ID was added as a random effect in the model to account for non-independence of observations within each cow.

For logistic regression models, odds ratios were calculated by exponentiating the coefficients from the model, and Wald 95% confidence intervals were determined using the '*Confint'* function from the 'car' package (Fox et al., 2022). Adjusted probabilities (1/(1+odds)) were estimated using the 'emmeans' package (Lenth et al., 2022). Multiple comparisons were accounted for using Tukey adjustment as implemented in the 'emmeans' package. The amount of variability explained by the random effects was assessed by estimating the intraclass correlation coefficient as implemented in the 'performance' package (Lüdecke et al., 2021). In some instances, the estimated variance of random effects was zero, making it impossible to compute the intraclass correlation coefficient, which was denoted as not available.

3.4. RESULTS

3.4.1 Herd characteristics

Herd characteristics for enrolled farms are shown in **Table 3.1**. Enrolled farms were in Colorado (N=1), Texas (N=1), Minnesota (N=2) and New Mexico (N=1). Herd size ranged from 100 and 3,000 milking cows. All the farms allowed access to pasture and cows consumed at least 30% of their dry-matter intake from pasture when possible. The housing systems differed between farms, with cows in Texas and New Mexico housed in dry lot pens; cows in Colorado and one Minnesota farm in a free stall barn; and cows in the other Minnesota farm having access to a compost barn and out-wintering lot during the winter.

3.4.2 Prevalence of any microorganisms

Among the enrolled cows, 84.7% (359/424) had an IMI during the postpartum period. The prevalence of IMI did not vary significantly across the postpartum period ($P = 0.25$) and was 59.2% (251/424) in sample 1, 56.4% (228/404) in sample 2, 60.9% (227/373) in sample 3, 54.2% (176/325) in sample 4, and 55.9% (85/152) in sample 5 (**Table 3.2**). The intraclass correlation coefficient suggested that 38.4% of the variability on the IMI prevalence was explained by cow-ID.

3.4.3. Staphylococcus aureus

During the postpartum period, 80 out of the 424 cows (18.9%) had a *Staphylococcus aureus*-IMI, with significant differences between farms ($P < 0.05$, **Figure 3.2**). For instance, in farm D none of the cows had a *Staphylococcus aureus*-IMI, whereas in farm E almost half of the cows (46.2%, 24/52) had a *Staphylococcus aureus*-IMI (**Figure 3.2**). The prevalence of *Staphylococcus aureus* remained relatively stable throughout the postpartum period in all enrolled farms, 14.6% (62/424), 13.6% (55/404), 13.9% (52/373), 13.4% (43/320) and 12.5% (19/152) in postpartum sample 1 to 5, respectively ($P = 0.86$, **Table 3.2**). *Staphylococcus aureus* was detected in the first sample after calving in 14.6% (62/424) of the animals, while the CmI was considerably lower (5.3%, 18/342, **Figure 3.2**). In fact, 76.6% (59/77) of *Staphylococcus aureus*-IMI during the postpartum period were already present in the first postpartum sample.

3.4.4. NASM

The PP of NASM-IMI was 52.1% (221/424) (**Figure 3.2**). Among NASM, *Staphylococcus chromogenes* was the most frequently isolated in all farms (**Figure 3.2**). The PP

of *Staphylococcus chromogenes*-IMI was 38.0% (161/424), with no statistically significant difference between farms (PP range: 31.5 to 48.1%, $P = 0.16$) or across the postpartum period ($P = 0.12$) (**Table 3.2**). At first sampling, 25.5% (108/424) of the animals had *Staphylococcus chromogenes*-IMI, while 17.9% (53/296) of the cows at risk acquired an IMI by this microorganism in subsequent samples (**Figure 3.3**). The percentage of *Staphylococcus chromogenes*-IMI harbored on the first sample was 66.2% (104/157). Other NASM (i.e., NASM non-*chromogenes*) showed a numerically lower PP (PP = 22.6% [96/424]) and divergent distribution across the enrolled farms ($P < 0.001$) (Figure 3). These microorganisms included: *Staphylococcus epidermidis* (PP = 0.2% [1/424]), *Staphylococcus haemolyticus* (PP = 2.1% [9/424]), *Staphylococcus hominis* (PP = 0.7% [3/424]), *Staphylococcus sciuri* (PP = 0.9% [4/424]), *Staphylococcus xylosus*/*saprophyticus* (PP = 2.4% [10/424]) and unspeciated *Staphylococcus spp.* (PP = 17.9% [76/424]). The prevalence of NASM non-*chromogenes* in the first postpartum sample was 7.1 % (30/424), and the CmI in the subsequent postpartum period was 17.6 % (66/374). (**Figure 3.3**). Only 27.5% (25/91) of the IMI caused by these microorganisms were found in the first postpartum sample.

3.4.5. SSLO

The PP of SSLO was 32.1% (136/424). Among SSLO, *Streptococcus* spp. had a PP of 16.7% (71/424) which differed significantly between farms ($P < 0.001$, **Figure 3.2**) and postpartum samples ($P = 0.008$) (**Table 3.2**). *Streptococcus* spp. were found at calving in 11.6% (39/424) of the enrolled animals and 6.2% (22/355) of the cows without *Streptococcus* spp.-IMI on the first sample acquired a new *Streptococcus* spp.-IMI during the following postpartum period (**Figure 3.3**). An important proportion of *Streptococcus* spp.-IMI during the postpartum

period (67.6% [37/61]) were already present in the first postpartum sample. *Streptococcus dysgalactiae* was the predominant species within the *Streptococcus* genus (PP: 11.1% [47/424]), while *Streptococcus uberis* (PP: 2.1% [9/424]) and unspeciated *Streptococcus* spp. (PP: 7.5% [32/424]) showed a numerically lower PP. The distribution of different species within the SSLO group varied across farms as shown in **Figure 3.2**. In our study, 17.0% (72/424) of the cows harbored SLO-IMI during early lactation. The prevalence of SLO varied significantly across farms ($P < 0.001$, **Figure 3.2**), but not between samples collected at different postpartum time-points ($P = 0.63$). The most prevalent species among this group included *Aerococcus* spp. (5.4% [23/424], and *Enterococcus* spp. (10.1% [43/424]), while *Lactococcus* spp. (1.4% [6/424]) and *Micrococcus* spp. (0.7% [3/424]) showed a low PP. Among the samples taken at calving, SLO species were found in 5.9% (25/424), whereas SLO-IMI CmI was 12.4% (47/379). SLO-IMI in the first sample represented 33.8% (24/71) of all SLO-positive samples.

3.4.6. Gram-negative microorganisms

Prevalence of gram-negative bacteria was significantly different between farms ($P < 0.001$, **Figure 2**). These microorganisms had a low PP in our study (8.7% [37/424]), with the exception of farm C that showed a statistically (vs. farm A and B) or numerically (vs. farm C and D) higher prevalence compared to other farms (PP: 20.7% [23/111]) (**Figure 3.2**). The predominant bacterial genus within gram-negative organisms was *Escherichia* spp. (PP = 3.3% [14/424]). *Klebsiella oxytoca*-IMI was identified only in Farm C and in very low frequency (PP = 0.7% [3/424]). Other gram-negative organisms isolated from milk samples in this study showed a low PP and included *Pseudomonas* spp. (0.7% [3/424]), *Enterobacter* spp. (0.5% [2/424]), *Pantoea* spp. (0.7% [3/424]), *Citrobacter freundii* (0.2% [1/424]), *Serratia* spp. (0.7%

[3/424]), and other unidentified gram-negative organisms (1.2% [5/424]). Gram-negative bacteria had a low prevalence throughout the follow-up period with no significant difference across postpartum samples ($P = 0.58$). Additionally, gram-negative microorganisms were harbored in 2.8% (12/424) of milk samples in the first postpartum sample, representing 32.4% (12/37) of gram-negative IMI, while 6.4% (25/392) of the cows at risk acquired a new gram-negative IMI during the subsequent postpartum period (**Figure 3.3**).

3.4.8. Others

'Others' comprise a diverse group of microorganisms that do not belong to any of the previously mentioned taxa. In this longitudinal study, 32.5% (138/424) of the animals acquired an IMI associated with this group during the follow-up period. The PP of IMI caused by microorganisms belonging to this group varied significantly across farms ($P < 0.001$, **Figure 3.2**) and ranged from 9.5% (Farm D, 2/21) to 72.1% (Farm C, 80/111). The most frequently isolated microorganisms in this group were *Bacillus* spp. (PP = 20.5% [87/424]) and *Corynebacterium* spp. (PP = 13.9% [59/424]). Other microorganisms from this group showed a very low prevalence during the study period (PP < 1%).

3.4.9. Persistence of intramammary infections

Associations between bacterial group and persistence of IMI were assessed to understand the epidemiology of the microorganisms associated with IMI in this observational study (**Figure 3.4**). *Staphylococcus aureus* exhibited high persistence, with 27.3% (21/77) and 59.7% (46/77) of the IMI caused by this microorganism being found in 2 and 3 or more samples, respectively. Similarly, 108 of the 157 (68.8%) cases of *Staphylococcus chromogenes* persisted for 2 or more

samples after calving. In contrast, the majority of NASM non-*chromogenes* caused transient-IMI and were only observed in 1 of the post-partum samples from each cow, i.e., 24.5% (24/98). For *Streptococcus* spp., 24 out of 45 (53.3%) *Streptococcus dysgalactiae*-IMI (the most frequently isolated SSLO in our study) were persistent. *Streptococcus uberis* and unspecified *Streptococcus* spp. had 50.0% (4/8) and 31.3% (10/32) of cases classified as persistent, respectively. Persistence for SLO-IMI was low, as these microorganisms showed predominantly short-lived IMI. Only 9.5% (8/84) of the SLO-IMI persisted for 2 or more samples. All IMI caused by gram-negative organisms were transient, as none of them were identified in more than 1 postpartum sample from the same cow. Other microorganisms not belonging to any of these groups also showed low persistence during the follow-up period (18.5%, 34 out of 184 IMI), including *Bacillus* spp. and *Corynebacterium* spp.

Mixed logistic regression modeling indicated that bacterial group ($P < 0.001$) and number of postpartum samples (OR [95%CI]: 1.80 [1.36-2.38], $P < 0.001$) were associated with the odds of IMI persistence (**Table 3.3**). Farm was not statistically significantly associated with IMI persistence ($P = 0.31$). After adjusting for multiple comparisons, *Staphylococcus aureus* (Adjusted (**Adj.**) risk [95%CI]: 0.89 [0.79-0.94]) showed a higher persistence than *Staphylococcus chromogenes* (Adj. risk [95%CI]: 0.72 [0.64-0.79]) ($P = 0.06$), while both microorganisms showed significantly higher odds of persistence compared to other bacterial groups: NASM non-*chromogenes* (Adj. risk [95%CI]: 0.26 [0.18-0.36], $P < 0.001$); *Streptococcus* spp. (Adj. risk [95%CI]: 0.44 [0.32-0.56], $P < 0.001$); SLO (Adj. risk [95%CI]: 0.10 [0.05-0.20], $P < 0.001$); other microorganisms (Adj. risk (95%CI): 0.20 [0.13-0.28], $P < 0.001$). Additionally, *Streptococcus* spp. showed a higher persistence compared to SLO (OR

[95%CI]: 6.67 [1.82-24.49], P <0.001), while no statistically significant difference was found between these groups and NASM non-*chromogenes* (P > 0.05).

3.5. DISCUSSION

This multi-site longitudinal study described the temporal prevalence dynamics and distribution of microorganisms associated with IMI in organically managed primiparous cows during early lactation. In our enrolled herds, 84.7% of the animals had an IMI during the postpartum period. The prevalence of IMI during the postpartum period remained relatively stable and ranged between 39.1% and 46.3% in the different postpartum samples (1st to 5th sample). This is comparable to the 48.7% prevalence in early lactation primiparous cows in conventional dairy farms in Europe (Piepers et al., 2010), and higher than that reported for quarter-level IMI in other epidemiological studies conducted in conventionally managed primiparous cows, which ranged from 14.2 % to 32.1% (Supré et al., 2011; Nitz et al., 2020; Valckenier et al., 2020).

3.5.1. Dynamics and distribution microorganisms leading to intramammary infections

In our study, the prevalence of *Staphylococcus aureus* was higher than that reported in other observational studies in conventionally managed first-lactation dairy cows, which ranges from 0.3% to 7.0 % (Piepers et al., 2010; Nitz et al., 2020; Valckenier et al., 2020). Indeed, previous studies have shown an increased prevalence of *Staphylococcus aureus* in organic compared to conventional dairies (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013) and may be associated with the restrictions placed on organic farms regarding the use of antimicrobials (NMC, 2019). Importantly, the majority of *Staphylococcus aureus*-IMI (76.6%) were already

present in the first sample after calving, in agreement with previous studies reporting a high prevalence of *Staphylococcus aureus*-IMI in primiparous cows before calving (0.4% to 14.9%) (De Vliegher et al., 2012). This suggests that prepartum management may play a role in the acquisition of *Staphylococcus aureus*-IMI (Phillips et al., 2021). The mechanisms that facilitate the transmission of *Staphylococcus aureus* to primiparous cows before calving are not completely elucidated. It has been suggested that potential pathways of transmission may involve the ability of *Staphylococcus aureus* to colonize other body sites (Anderson et al., 2012; De Vliegher et al., 2012), or be transmitted through vectors like flies (Gillespie et al., 1999). Although certain strains affecting primiparous cows could be of environmental origin (Zadoks et al., 2011), identical strains have been reported to affect multiparous and primiparous cows, suggesting that within herd transmission across these groups is plausible (Tenhagen et al., 2007).

Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species are the most frequently isolated microorganisms associated with IMI in conventionally managed first-lactation cows (De Buck et al., 2021). In our study, NASM were also the most prevalent microorganisms detected in milk samples in early lactation. Surprisingly, we did not identify a statistically significant association between postpartum sample and NASM-IMI, which contradicts previous studies that showed an increased prevalence of NASM-IMI in the first days after calving in comparison to the subsequent postpartum period (Valckenier et al., 2020). Among identified NASM species, *Staphylococcus chromogenes* was the most frequently isolated in all enrolled farms, consistent with reports from conventional dairy farms (De Buck et al., 2021). In previous epidemiological studies, the prevalence of *Staphylococcus chromogenes* in first-lactation cows ranged between 5.5% and 13% (Supré et al., 2011; De Visscher et al., 2016a;

Valckenier et al., 2020) and was higher in first-lactation compared to multiparous cows (De Visscher et al., 2016a; Nyman et al., 2018). Other NASM species (i.e., NASM non-*chromogenes*) showed a lower prevalence overall, with differences in prevalence across the enrolled farms. It has been reported that most of these NASM species have a poor adaptation to the mammary gland and a predominantly environmental epidemiology (De Buck et al., 2021). For example, previous studies reported that these microorganisms showed a higher prevalence in environmental niches compared to milk samples (De Visscher et al., 2014; Wuytack et al., 2020), which may potentially explain the lower prevalence and differential distribution of these microorganisms across farms (De Buck et al., 2021). In general, differences in microorganism prevalence between the farms in our study was not surprising given the numerous differences in management, including bedding materials, housing systems and/or geographical regions of farms that were enrolled in our study (**Table 3.1**).

The SSLO are an important cause of clinical mastitis and a concern to dairy farmers around the world (Kabelitz et al., 2021). In the present observational study, the prevalence of SSLO was higher than that reported in conventionally managed first-lactation animals (0.6% - 5.0%) (Piepers et al., 2010; Nitz et al., 2020; Valckenier et al., 2020). *Streptococcus* spp. were the most prevalent genus within SSLO. The PP of *Streptococcus* spp. showed important differences across farms, suggesting that differential management and housing systems could have an impact on its prevalence. Following a similar pattern to *Staphylococcus aureus*, Farm B and E showed a higher prevalence of *Streptococcus* spp. compared to the other farms. This suggests a predominance of pathogens adapted to the mammary gland. Indeed, organic dairy farms face challenges to deal with host-adapted microorganisms due to the restrictions placed on

the use of antimicrobials and the potential difficulties finding replacements for culled animals

(NMC, 2019). Among *Streptococcus* spp., *Streptococcus dysgalactiae* was the most prevalent.

Streptococcus dysgalactiae is recognized as an important mastitis pathogen, usually classified as

an intermediate pathogen (Kabelitz et al., 2021), in which environmental and host-associated

strains are frequently present within a dairy farm (Wente and Krömker, 2020). The SLO

represents a diverse group of microorganisms that are commonly misdiagnosed as *Streptococcus*

spp. While these microorganisms are thought to be environmental (Hogan and Smith, 2012),

little is known about the epidemiology of different species within this group. The SLO-IMI have

been associated with an increase in the risk of subclinical mastitis, indicating their importance

for udder health (Rowe et al., 2021).

Gram-negative microorganisms are an important cause of clinical mastitis in

conventionally managed dairy cows (Lago et al., 2011; Royster et al., 2014). However, the

prevalence of gram-negative bacteria is usually reported to be low (0% - 8.5%) (Piepers et al.,

2010; Nitz et al., 2020; Valckenier et al., 2020), due to the poor adaptation of gram-negative

microorganisms to the mammary gland (Todhunter et al., 1991; Klaas and Zadoks, 2018). Only

8.7% of the enrolled cows had gram-negative-IMI, showing a similar low prevalence in all farms

except for farm C. Consistently, farm C also showed a higher prevalence of SLO. While the

specific factors that led to these differences are unknown, it is possible that factors related to the

housing system had an impact on the prevalence of these microorganisms in farm C. Dry-lots are

thought to increase cow exposure to environmental hazards such as rain, wind or sun, since cows

are less sheltered than in indoor confinement systems. This higher exposure can impact animal

health and welfare (Chen et al., 2017), which could partially explain the higher prevalence of

environmental microorganisms in farm C. However, if dry-lot housing was the primary driver we would also expect similar dynamics in farm B, but that was not the case, suggesting the presence of other unknown factors that may have influenced the prevalence of gram-negative bacteria in the different farms of this study.

3.5.2. Persistence of intramammary infections

The prevalence of IMI at any given time point represents both acquisition of new IMI and IMI persistence. Different strategies can be used in both organic and conventional dairy farms to reduce the risk of new IMI (Ruegg, 2017; NMC, 2019). Nonetheless, antibiotics are the most frequently used strategy in conventional dairy farms for managing IMI after they are acquired (Halasa et al., 2009a; Ruegg, 2021); this strategy is not available to certified organic dairies unless the treated cows are removed from the herd (NMC, 2019). Our study highlights how IMI persistence varies among different bacterial species. These differences may have implications for the epidemiology of mastitis-causing microorganisms, prognosis of infected quarters and decision-making in dairy farms; and these results are especially crucial for organic-certified dairy farms in which antibiotics are not used for control and herd-level management of mastitis. Not surprisingly, a large proportion of *Staphylococcus aureus*-IMI in our study were persistent (Adj. risk: 89%), conforming to the idea that this bacterium has high adaptation to the mammary gland and an ability to persist within it for long periods of time (Barkema et al., 2006). *Staphylococcus chromogenes* also showed a high persistence in the mammary gland (Adj. risk: 72%) which agrees with previous reports (Piessens et al., 2011; Supré et al., 2011; Vanderhaeghen et al., 2015). *Streptococcus* spp. showed a moderate likelihood of persistence in the mammary gland, with almost half of the IMI being classified as persistent (Adj. risk: 44%). This could be

attributed to the fact that *Streptococcus dysgalactiae*, the most prevalent *Streptococcus* species in our study, is known for its ability to survive in the mammary gland and persist for long periods (Calvinho and Oliver, 1998; Kabelitz et al., 2021), although some strains can be environmental (Wente and Krömker, 2020). In contrast to *Streptococcus* spp. a low proportion of SLO-IMI were persistent, suggesting a poor adaptation to the mammary gland, which agrees with their reported environmental epidemiology (Hogan and Smith, 2012). All of the gram-negative-IMI were only present in the mammary gland for 1 postpartum sample (i.e., transient-IMI), which can be explained by the poor host adaptation and predominantly environmental epidemiology of gram-negative microorganisms (Todhunter et al., 1991; Klaas and Zadoks, 2018). The exception to this is *Klebsiella* spp., which had low prevalence in the present study (detected in 3 out of 424 animals).

3.5.3. Study limitations and internal validity

The prevalence and distribution of microorganisms associated with early lactation IMI in organic dairy farms described in our study does not agree with previous reports from conventional dairy farms. Our study was not designed to identify the drivers of these differences, and many factors could be responsible for these findings. Some of these factors may be related to organic production practices (such as antimicrobial use, as discuss), but others could be due to differential cow demographics and management between organic and conventional farms. For example, it has been reported that organic farms have a higher average parity (Hardeng and Edge, 2001; Stiglbauer et al., 2013) which is associated with an increased prevalence of contagious mastitis pathogens and particularly *Staphylococcus aureus* (De Vliegher et al., 2012; Ziesch and Krömker, 2016), that could potentially be transmitted to first-lactation cows before

and after calving (De Vliegher et al., 2012; Dufour et al., 2012). Furthermore, organic dairies are less likely to routinely measure SCC, which may lead to subpar identification of chronically infected cows that can play a key role in the dissemination of IMI organisms (Stiglbauer et al., 2013). The management and environment in which cows are raised also has an impact on udder health. The possible exposure to mastitis bacteria and the environment can be assessed using the udder hygiene score (Schreiner and Ruegg, 2003), which has been reported to be lower in organic farms (Ellis et al., 2007). Antimicrobials and teat sealants can still be used to safeguard udder health on conventionally maintained cows during the dry period (Berry and Hillerton, 2007; Halasa et al., 2009b), but their use is not permitted under organic management (NMC, 2019).

One important strength of this study is the longitudinal approach of sampling the same animals multiple times after calving, which allows for the investigation of not only IMI prevalence, but also the likelihood of IMI acquisition (incidence) and IMI persistence during the first 5 weeks of lactation. In addition, the use of MALDI-TOF MS provides a higher taxonomic resolution than traditionally utilized biochemical methods (Royster et al., 2014), allowing species-level identification for many of the isolates (Jahan et al., 2021). Limitations of this study include the potential misclassification of true IMI as contaminated samples due to mixed infections across quarters (Dean et al., 2022), that could result in an underestimation of the true IMI prevalence. Likewise, the use of composite milk samples could lead to an underestimation of the prevalence of IMI due to a dilution effect (Reyher and Dohoo, 2011). In an effort to overcome these constraints, this study utilized an IMI definition of 10 CFU/mL of any culturable isolate in contrast to the commonly used 100 CFU/mL, hence increasing the likelihood of

detecting an IMI in the processed milk samples (Dohoo et al., 2011). In addition, contamination was defined as more than 3 different isolates in a given sample in order to reduce the misclassification of mixed inter-quarter infections as contaminated milk samples (Dean et al., 2022). The absence of strain-typing techniques in this study represents another limitation for the investigation of IMI persistence (Simar et al., 2021). This could lead to an IMI being categorized as persistent when, in fact, different strains or species within the same taxonomic group could be causing an infection across multiple sampling points. Therefore, our results could be potentially overestimating the persistence of IMI in such cases. Lastly, in our study, MALDI-TOF MS failed to identify a considerable number of isolates at the species level. For instance, 78.4% (422/538) of NASM isolates were identified up to the species level, which is consistent with previous studies (Rowe et al., 2019, 2020). The taxonomic resolution for the identification of these microorganisms may have been enhanced by using lower confidence score thresholds, such as 1.7 for taxonomic assignment at the species level, as previously examined (Cameron et al., 2017). As an example, we still had access to data from a small proportion of the isolates processed using MALDI-TOF MS that were not speciated (10.3% [12/116]). Among these isolates, 3 (25%) could have been identified up to the species level if the cut-off value for MALDI-TOF MS identification had been lowered to 1.7. These isolates would have been identified as *Staphylococcus xylosus*, *Staphylococcus haemolyticus* and *Staphylococcus warneri*. However, in order to be consistent with the manufacturer recommendation and enable comparisons between this and other studies performed in North America we decided to utilize a 2.0 confidence score for species level identification (Jahan et al., 2021). Nonetheless, to overcome the limited ability to identify the NASM and other mastitis microorganisms species

using MALDI-TOF MS, the use of alternate cut-off points (Cameron et al., 2017) and the use of enlarged databases (Cameron et al., 2018) should be considered to promote reproducibility across and within research institutions.

3.5.4. External validity

This study presents results for 5 organic dairy farms in the Midwest and Southwest regions, including farms with large and small herd sizes. Therefore, we consider our study findings relevant to a diversity of organic dairy farms in the United States. However, it is worth noting that only a few of the farms contacted by the research team agreed to participate in this study. Since the underlying reasons that led to participation are unknown, this could potentially lead to a type of selection bias known as non-response bias (Groves and Peytcheva, 2008). In addition, more studies are needed to investigate IMI dynamics in both primiparous and multiparous dairy cows in a larger number of organic dairy farms, in order to identify potential farm-level risk factors that could be driving the observed differences in the prevalence and distribution of mastitis pathogens between the enrolled herds.

3.6. CONCLUSIONS

This study provides a detailed description of the microorganisms leading to IMI in early lactation first-lactation dairy cows on organic dairy farms. Our results showed a high prevalence of *Staphylococcus aureus*, NASM and SSLO in the enrolled animals during the start of their first lactation. The prevalence of these microorganisms differed by herd. Additionally, certain bacterial microorganisms, especially *Staphylococcus aureus* and *Staphylococcus chromogenes*, showed a high prevalence at calving and high persistence in the mammary gland, which suggests

that prepartum management should be a focus of IMI prevention and control in organically

reared primiparous cows. Lastly, some IMI species showed a predominance of persistent-IMI

(e.g., *Staphylococcus aureus*, *Staphylococcus chromogenes* and *Streptococcus* spp.), while others

caused primarily transient-IMI (e.g., NASM non-*chromogenes*, SLO, gram-negative

microorganisms). This study allows us to better understand the epidemiology of mastitis-causing

pathogens in organically managed first-lactation cows, which is a necessary step towards

developing tailored mastitis controls for this unique and growing subpopulation of U.S. dairy

cows.

3.7. ACKNOWLEDGMENTS

3.8. TABLES AND FIGURES

3.8.1. Tables

Table 3.1. Herd characteristics in each of the enrolled herds (A–E). A total of 503 first-lactation dairy cows from 5 organic dairy farms are included in this table.

Farm	Herd size	Number enrolled cows	Location	Housing system
A	1700	162	Colorado	Free stall barn with exercise pen + Grazing
B	3000	122	Texas	Dry lot + Grazing
C	1500	130	New Mexico	Dry lot + Grazing
D	100	23	Minnesota	Free stall barn + Grazing
E	275	66	Minnesota	Compost Barn + Outwintering lot + Grazing

Table 3.2. Culture results by postpartum sample (PS). A total of 1673 composite milk samples from 424 cows are included in this table.

Culture Result	PS 1 (n=424)	PS 2 (n=404)	PS 3 (n=373)	PS 4 (n=320)	PS 5 (n=152)	*P-value* †
Staph. aureus	62 (14.6%)	55 (13.6%)	52 (13.9%)	43 (13.4%)	19 (12.5%)	0.86
NASM	132 (31.1%)	127 (31.4%)	116 (31.1%)	79 (24.7%)	44 (28.9%)	
Staph. chromogenes	108 (25.5%)	91 (22.5%)	92 (24.7%)	63 (19.7%)	33 (21.7%)	0.12
NASM non-*chromogenes*	30 (7.1%)	42 (10.4%)	31 (8.3%)	21 (6.6%)	13 (8.6%)	0.50
Staph. epidermidis	0 (0%)	0 (0%)	1 (0.3%)	0 (0%)	0 (0%)	
Staph. haemolyticus	0 (0%)	3 (0.7%)	2 (0.5%)	3 (0.9%)	1 (0.7%)	
Staph. hominis	0 (0%)	1 (0.2%)	2 (0.5%)	0 (0%)	0 (0%)	
Staph. sicuri	1 (0.2%)	1 (0.2%)	1 (0.3%)	1 (0.3%)	0 (0%)	
Staph. xylosus/saprophyticus	3 (0.7%)	4 (1.0%)	2 (0.5%)	1 (0.3%)	0 (0%)	
Staph. sp.	26 (6.1%)	35 (8.7%)	24 (6.4%)	16 (5.0%)	12 (7.9%)	
SSLO	74 (17.5%)	58 (14.4%)	53 (14.2%)	37 (11.6%)	14 (9.2%)	
Strep. spp.	49 (11.6%) [a]	36 (8.9%) [ab]	31 (8.3%) [ab]	24 (7.5%) [b]	8 (5.3%) [b]	0.008
Strep. dysgalactiae	33 (7.8%)	17 (4.2%)	17 (4.6%)	17 (5.3%)	5 (3.3%)	
Strep sp.	10 (2.4%)	17 (4.2%)	10 (2.7%)	5 (1.6%)	2 (1.3%)	
Strep. uberis	6 (1.4%)	2 (0.5%)	4 (1.1%)	3 (0.9%)	1 (0.7%)	
Strep.-like organisms	25 (5.9%)	23 (5.7%)	23 (6.2%)	13 (4.1%)	6 (3.9%)	0.63
Aerococcus viridans	10 (2.4%)	5 (1.2%)	6 (1.6%)	4 (1.3%)	0 (0%)	
Aerococcus sp.	0 (0%)	3 (0.7%)	1 (0.3%)	0 (0%)	0 (0%)	
Enterococcus casseliflavus	6 (1.4%)	3 (0.7%)	2 (0.5%)	2 (0.6%)	1 (0.7%)	
Enterococcus faecalis	0 (0%)	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	
Enterococcus hirae	3 (0.7%)	2 (0.5%)	2 (0.5%)	1 (0.3%)	1 (0.7%)	
Enterococcus mundtii	2 (0.5%)	3 (0.7%)	2 (0.5%)	3 (0.9%)	0 (0%)	
Enterococcus saccarolyticus	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0.7%)	
Enterococcus sp.	5 (1.2%)	6 (1.5%)	7 (1.9%)	1 (0.3%)	3 (2.0%)	
Lactococcus garviae	1 (0.2%)	1 (0.2%)	0 (0%)	1 (0.3%)	0 (0%)	
Lactococcus lactis	0 (0%)	0 (0%)	2 (0.5%)	1 (0.3%)	0 (0%)	
Lactococcus sp.	0 (0%)	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	
Micrococcus sp.	1 (0.2%)	1 (0.2%)	1 (0.3%)	0 (0%)	0 (0%)	
Gram-negative bacteria	12 (2.8%)	10 (2.5%)	10 (2.7%)	5 (1.6%)	2 (1.3%)	0.58
Citrobacter freundii	0 (0%)	0 (0%)	0 (0%)	1 (0.3%)	0 (0%)	
Enterobacter clocae	0 (0%)	0 (0%)	1 (0.3%)	0 (0%)	0 (0%)	
Enterobacter sp.	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Escherichia coli	6 (1.4%)	5 (1.2%)	2 (0.5%)	0 (0%)	0 (0%)	
Escherichia sp.	2 (0.5%)	0 (0.0%)	1 (0.3%)	0 (0%)	0 (0%)	
Klebsiela oxytoca	0 (0%)	0 (0%)	1 (0.3%)	2 (0.6%)	0 (0.0%)	
Pantoea sp.	1 (0.2%)	1 (0.2%)	1 (0.3%)	0 (0%)	0 (0%)	
Pseudomonas sp.	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Serratia sp.	0 (0%)	1 (0.2%)	1 (0.3%)	0 (0%)	1 (0.7%)	
Other gram-negative	2 (0.5%)	0 (0%)	1 (0.3%)	1 (0.3%)	1 (0.7%)	
Other	40 (9.4%)	46 (11.4%)	54 (14.5%)	43 (13.4%)	22 (14.5%)	0.44
Arthrobacter gandaviensis	1 (0.2%)	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	
Athrobacter sp.	0 (0%)	1 (0.2%)	0 (0%)	0 (0%)	0 (0%)	
Bacillus sp.	18 (4.2%)	28 (6.9%)	27 (7.2%)	25 (7.8%)	10 (6.6%)	
Corynebacterium sp.	12 (2.8%)	15 (3.7%)	20 (5.4%)	17 (5.3%)	7 (4.6%)	
Helcococcus ovis	0 (0%)	0 (0%)	1 (0.3%)	0 (0%)	0 (0%)	
Other gram-positive cocci	1 (0.2%)	4 (1.0%)	2 (0.5%)	4 (1.3%)	3 (2.0%)	
Other gram-positive rod	6 (1.4%)	1 (0.2%)	7 (1.9%)	4 (1.3%)	2 (1.3%)	
Trueperella pyogenes	1 (0.2%)	1 (0.2%)	1 (0.3%)	0 (0%)	0 (0%)	
Yeast	1 (0.2%)	0 (0%)	1 (0.3%)	0 (0%)	0 (0%)	
No growth	173 (40.8%)	176 (43.6%)	146 (39.1%)	148 (46.3%)	67 (44.1%)	0.25

PS: Postpartum sample. NASM: Non-aureus *Staphylococci* and closely related *Mammaliicoccal* species. SSLO: *Streptococcus* spp. and *Streptococcus*-like organisms. † Type-III *P-value* assessing the association between the postpartum sample and presence of IMI by a specific microorganism, accounting for farm-ID and cow-ID as a random effect. Different superindices within the same row represent significant differences across different postpartum samples. All models accounted for farm-ID as a fixed effect and cow-ID as a random effect. Intraclass correlation coefficient Cow-ID: Any-IMI = 0.38, *Staphylococcus aureus*-IMI = 0.98, *Staphylococcus chromogenes*-IMI = 0.85, NASM non-*chromogenes*-ID = 0.48 *Streptococcus* spp.-IMI = 0.93, *Streptococcus*-like organisms-IMI = 0.30, Gram-negative bacteria-IMI = Not available, Others-IMI = 0.14.

Table 3.3. Mixed logistic regression model assessing the association between Bacterial group (Explanatory variable) and odds of persistent infection (Harbor same microorganism in milk in 2 or more milk samples during follow-up period). Each observation in this model represents one IMI (n= 685).

Variable	n	Adj. Risk (95%CI))	Estimate (SE)	OR (95% CI)	*P-value*
Bacterial group †					<0.001
Staphylococcus aureus	77	0.89 (0.79-0.94) [a]	*Referent*	*Referent*	
Staphylococcus chromogenes	157	0.72 (0.64-0.79) [b]	-1.09 (0.39)	0.34 (0.16-0.72)	
NASM *non-chromogenes*	98	0.26 (0.18-0.36) [cd]	-3.10 (0.43)	0.05 (0.02-0.10)	
Streptococcus spp.	85	0.44 (0.32-0.56) [c]	-2.29 (0.42)	0.10 (0.04-0.23)	
Streptococcus-like organisms	84	0.10 (0.05-0.20) [d]	-4.19 (0.53)	0.02 (0.01-0.04)	
Other organisms	184	0.20 (0.13-0.28) [cd]	-3.46 (0.42)	0.03 (0.01-0.07)	
Num. samples per cow	-	-	0.59 (0.14)	1.80 (1.36-2.38)	<0.001
Farm					0.31
A	95	0.42 (0.30-0.55)	*Referent*	*Referent*	
B	198	0.37 (0.29-0.47)	-0.21 (0.33)	0.81 (0.42-1.57)	
C	235	0.34 (0.26-0.44)	-0.34 (0.34)	0.71 (0.36-1.39)	
D	42	0.46 (0.28-0.65)	0.15 (0.47)	1.17 (0.46-2.94)	
E	115	0.52 (0.40-0.65)	0.42 (0.35)	1.51 (0.76-3.00)	

NASM non-chromogenes: *Staphylococcus epidermidis, Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus sciuri*, *Staphylococcus xylosus/saprophyticus*, *Staphylococcus* spp. *Streptococcus* like organisms: *Enterococcus* spp., *Aerococcus* spp., *Lactococcus* spp., *Micrococcus* spp. Cow-ID was added as a random effect (Variance [SD]: 1.88 e-14 [1.37 e^{-7}], Intraclass correlation coefficient = Not available). Gram negative organisms-IMI (n = 36) were not included in this model, because they had zero cases of persistent-IMI. † Different superindices within the same column represent significant differences across bacterial groups.

3.8.2. Figures
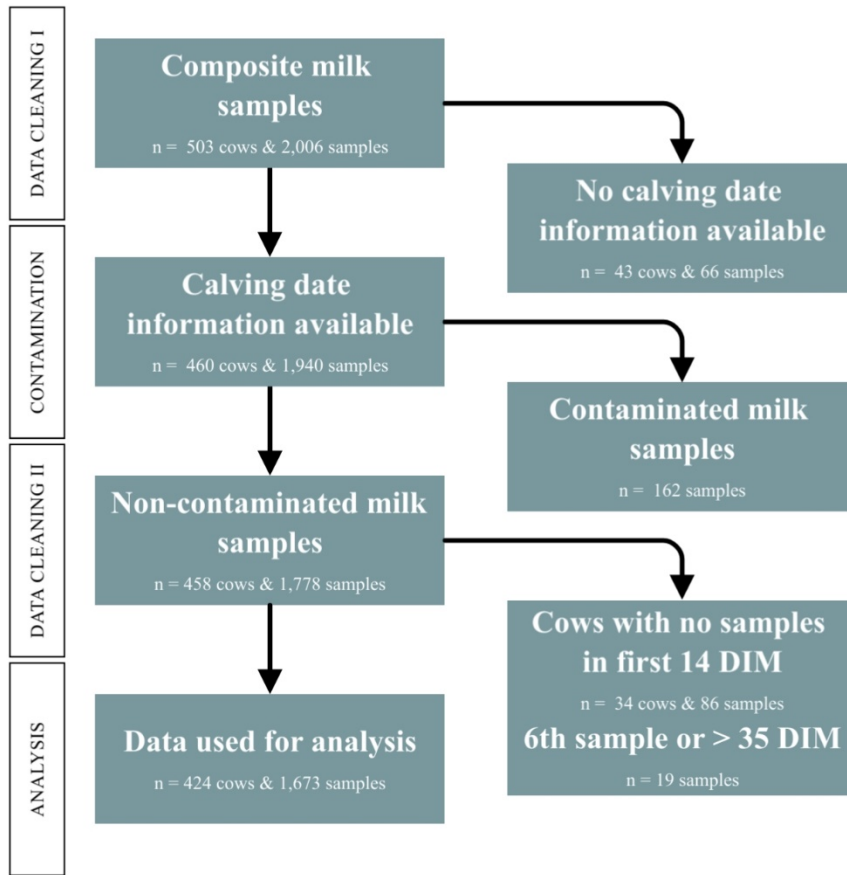
Figure 3.1. Flowchart illustrating the number of composite milk samples and cows excluded from the analysis during the data cleaning process. The left side outlines each cleaning step, while the central flowchart depicts the number of cows and samples at each step of the data cleaning process. On the right, the number of excluded cows and samples, along with the reasons are shown.
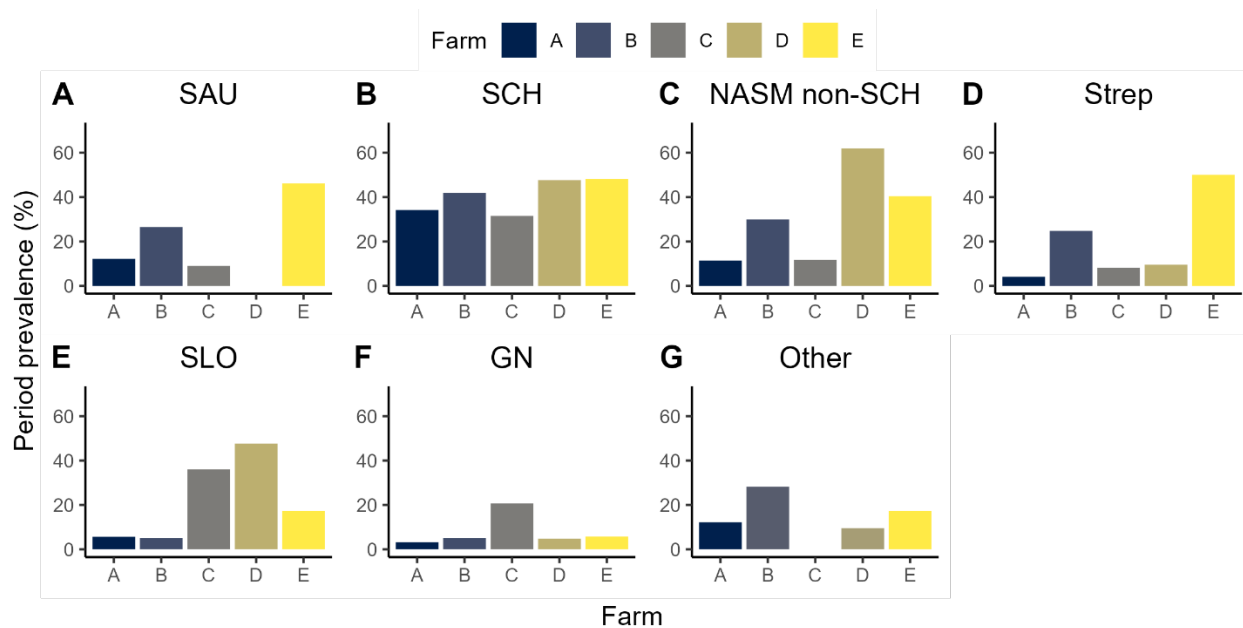
Figure 3.2. Period prevalence by taxonomic group and farm (n=424). A: SAU = *Staphylococcus aureus* (Type III *P*-value < 0.001). B: SCH = *Staphylococcus chromogenes* (Type III *P*-value = 0.16). C: NASM non-SCH = Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species that were not *Staphylococcus chromogenes* (Type III *P*-value < 0.001). D: Strep = *Streptococcus* species. E: SLO = *Streptococcus*-like organisms. F: GN = Gram-negative bacteria (Type III *P*-value < 0.001). G: Other = Other microorganisms not included in the previous groups (Type III *P*-value < 0.001).
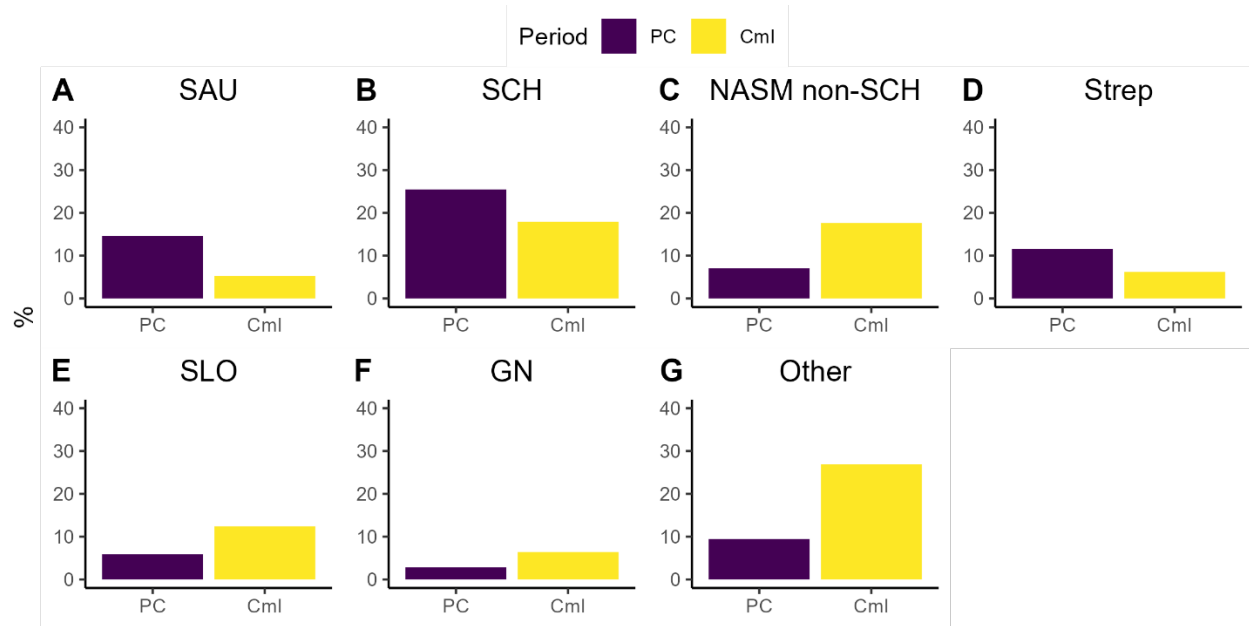
Figure 3.3. Prevalence in first postpartum sample (PC) and cumulative incidence (CmI) by taxonomic group (n=424). A: SAU = *Staphylococcus aureus*. B: SCH = *Staphylococcus chromogenes*. C: NASM non-SCH = Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species that were not *Staphylococcus chromogenes*. D: Strep = *Streptococcus* species. E: SLO = *Streptococcus*-like organisms. F: GN = Gram-negative bacteria. G: Other = Other microorganisms not included in the previous groups.
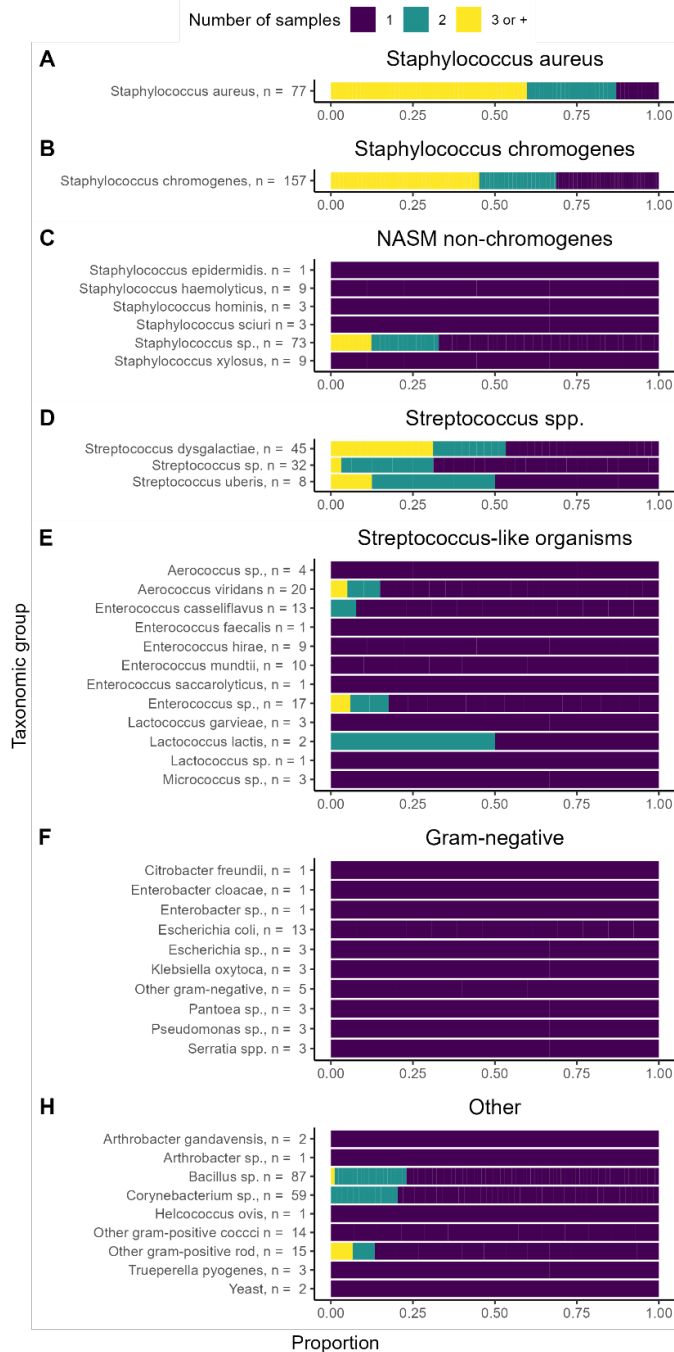
Figure 3.4. Persistence in the mammary gland by bacterial group (n=685). Data from 404 cows was included in this figure. Number of samples: number of samples that the same microorganism from a bacterial group was harbored during the postpartum period. NASM non-*chromogenes* = Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species that were not *Staphylococcus chromogenes*. Other = Other microorganisms not included in the previous groups.

CHAPTER 4: ASSOCIATIONS BETWEEN EARLY LACTATION INTRAMAMMARY INFECTIONS AND UDDER HEALTH AND PERFORMANCE DURING THE FIRST 180 DAYS IN MILK IN FIRST-LACTATION ORGANIC DAIRY COWS

4.1 SUMMARY

Prior data from our group showed that first-lactation cows under organic management in United States have a high prevalence of *Staphylococcus aureus*, *Streptococcus* spp. and *Staphylococcus chromogenes*-intramammary infections (**IMI**) in early lactation. Nonetheless, the relationship between IMI, udder health and milk production in organically reared primiparous cows remains elusive. The objectives of this observational study were to investigate the relationship between presence and persistence of IMI in the first 35 days in milk (**DIM**) and somatic cell count (**SCC**) and milk production during the first 6 months of lactation on first-lactation organic dairy cows. The analysis included a total of 1,348 composite milk samples collected during the first 35 DIM that were submitted for milk culture and 1,674 DHIA tests during the first 180 DIM from 333 heifers in 4 organic dairy farms, enrolled between February 2019 and January 2020. The association between IMI in the first 35 DIM and new high SCC (SCC>200,000 cells/mL) and milk production during the first 6 months of lactation was investigated using Cox proportional hazards regression and mixed linear regression, respectively. The association between IMI persistence (harboring the same microorganism as reported by the laboratory for 2 or more samples) in the first 35 DIM and number of DHIA tests with high SCC during the first 6 months of lactation was modeled using negative binomial regression. The presence of IMI by *Staphylococcus aureus* (HR [95%CI]): 3.35 [2.64, 4.25]) or *Streptococcus* spp. (HR [95%CI]: 2.25 [2.12, 2.39]) during the first 35 DIM was associated with an increased risk of new high SCC during the first 6 months of lactation. Milk production was reduced when *Streptococcus* spp. were identified in milk samples. However, there was no evidence of a difference in milk production in *Staphylococcus aureus*-IMI. Isolation of non-*aureus*

Staphylococci and closely related *Mammaliicoccal* species was related to a mild increase in the hazards of high SCC (HR [95%CI]: 1.34 [0.97, 1.85]) and a decrease in milk production during 1 or more postpartum tests. Presence of gram-negative or *Streptococcus*-like organisms-IMI was not associated with either high SCC or milk production. Presence of *Bacillus*-IMI was associated with a lower hazard of new high SCC (HR [95%CI]: 0.45 [0.30, 0.68]), and higher milk production during the first 180 days of lactation (overall estimate [95%CI]: 1.7 kg/day [0.3, 3.0]). The persistence of IMI in the first 35 DIM was associated to the number of tests with high SCC during the lactation for all microorganisms except for *Staphylococcus chromogenes*. Therefore, our results suggest that the persistence of IMI in the first 35 DIM could be an important factor to understand the association between IMI detected in early lactation and lactational SCC and milk production in organic dairy heifers. Our study described associations between IMI, udder health, and milk production in first-lactation organic dairy cows that are consistent with findings from conventional dairy farms.

4.2. INTRODUCTION

Mastitis remains one of the most costly and difficult diseases to control on dairy farms. While multiple strategies exist to prevent the acquisition of new intramammary infections (**IMI**) (Ruegg, 2017). Antibiotic therapy remains one of the most effective tools to treat and control contagious mastitis in conventional dairy farms (Halasa et al., 2009a; Ruegg, 2017). Organic dairy farms, however, face additional challenges to control mastitis given the restrictions placed on the use of antibiotics (NMC, 2019). Previous reports have described an increased prevalence of *Staphylococcus aureus* (**SAU**) in bulk tank milk samples of organic compared to conventional

dairy farms. (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). Nonetheless, information regarding the distribution of other microorganisms on organic dairy farms is scarce. Given the well-documented potential for negative impacts of IMI on udder health and productivity on conventional dairies (De Vliegher et al., 2012; Valckenier et al., 2020), it may be useful to enhance our understanding of the relationship between presence and persistence of IMI on organic dairy farms. Considering the restrictions imposed on the use of antibiotics in organic dairy farms, it is conceivable that these restrictions might enhance the ability of mastitis pathogens to persist within the mammary gland. This could potentially amplify the adverse effects on udder health, particularly for pathogens adapted to the udder environment. Consequently, more research into the relationship between IMI and udder health in organic dairy farms is warranted.

A subpopulation of cows of particular interest are first-lactation dairy cows, since herd replacement costs are even higher in organic compared to conventional dairies (McBride and Greene, 2007). While antibiotics are not typically administered to first-lactation cows for treatment of IMI in conventional dairy farms before calving, their use during lactation or the dry period in conventional farms could impact the overall herd-level prevalence of contagious mastitis pathogens (Ruegg, 2021; McCubbin et al., 2022; Dziuba et al., 2023). Consequently, first-lactation cows in organic farms, where antibiotics are prohibited (NMC, 2019), may be indirectly at a higher risk of IMI before or around calving due to the potential increased mastitis pathogen prevalence (De Vliegher et al., 2004b; Anderson et al., 2012; Dufour et al., 2012). A previous article published by our group (Peña-Mosca et al., 2023b) reported that SAU, *Staphylococcus chromogenes* (**SCH**) and *Streptococcus* spp. (***Strep***) were the most prevalent

mastitis-causing microorganisms in organic primiparous cows during the first month of lactation. These microorganisms were identified in 46.2%, 48.1% and 50% of the primiparous dairy cows in organic farms, respectively and showed a high IMI persistence. Our observation of infection persistence is consistent with studies from conventional dairy farms in which these same bacterial microorganisms had a high adaptation to the mammary gland, although differences do exist across and even within species (Keane, 2019; De Buck et al., 2021; Kabelitz et al., 2021). For instance, *Streptococcus dysgalactiae* is frequently classified as an "intermediate" pathogen (i.e., can be a contagious or environmental mastitis pathogen) (Wente and Krömker, 2020; Kabelitz et al., 2021), while *Streptococcus uberis*-IMI are predominantly transient and environmental (Wente et al., 2019; Leelahapongsathon et al., 2020). In addition, our findings of IMI persistence offer a potential explanation for previous reports that high SCC persisted throughout and across lactations in organic dairies (Fernandes et al., 2021). All the above suggest a predominance of udder-adapted mastitis pathogens in first-lactation organic dairy cows, raising concerns about the potential impact on udder health and performance during subsequent lactations.

In conventionally reared primiparous cows, the relationship between presence of IMI, udder health and performance varies across different microorganisms. Certain microorganisms are considered to be major pathogens (SAU, *Strep* and coliforms) that have a clear negative impact on udder health and performance during the subsequent lactation (Keane, 2019). Meanwhile, non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (**NASM**) are considered to be minor pathogens, and in some studies are associated with improved milk production in the subsequent lactation (Compton et al., 2007a; Piepers et al., 2010, 2013).

However, contradictory reports suggest that the pathogenicity of these microorganisms are species or strain-dependent (De Buck et al., 2021). Nonetheless, the relationship between IMI, udder health and milk production remain unclear and further research in organically reared primiparous cows is needed.

The primary objective of this study was to investigate the relationship between presence of IMI by a specific microorganism during the first 35 days in milk (**DIM**), and SCC and milk production during the first 180 DIM in first-lactation organic dairy cows. We hypothesized that the presence of major IMI pathogens would be associated with increased risk of new high SCC and lower milk production throughout the lactation. In contrast, we hypothesized that the presence of minor IMI pathogens would be associated with a lower risk of new high SCC and increased milk production during the first 6 months of lactation compared to cows without an IMI by these microorganisms. Our secondary objectives were to investigate the association between persistence of IMI in the first 35 DIM and the number of DHIA tests with high SCC throughout the first 180 DIM in these cows. We hypothesized that persistent IMI by a specific microorganism would be associated with a higher number of DHIA tests with high SCC during the first 180 days of lactation, while the presence of transient IMI by that microorganism would not be related to a significant increase in the number of DHIA tests with high SCC during the first 180 DIM compared to cows without an IMI by a specific microorganism.

4.3. MATERIALS AND METHODS

This longitudinal observational study included 503 nulliparous dairy cows in 5 organic dairy farms. Cows were enrolled 8 weeks prior to their first calving and followed up to 180 DIM.

All study activities were approved by the University of Minnesota Institutional Animal Care and Use Committee (**IACUC**) (Protocol Number: 1807: 36109A), Colorado State University IACUC (Protocol number: 1442) and Texas Tech University IACUC (Protocol number: 18068-10).

4.3.1. Inclusion criteria

This study is part of a larger research initiative to investigate potential associations between the udder microbiome and udder health (Dean et al., 2021). Only animals from USDA organic certified dairy farms were enrolled in this study. Different sized farms were selected from multiple United States regions. Enrollment was based on willingness to participate in the study, and proximity to the universities involved in the study. During the enrollment process, we attempted to include farms that possessed electronic farm records. However, to maintain a balanced representation of both small and large dairy farms in the study, we ultimately included a small farm even though it lacked farm records. For this longitudinal observational study, we enrolled 503 nulliparous cows from 5 organic dairy farms (Farm A: n=162, Farm B: n=122, Farm C: n=130, Farm D: n=23, Farm E: n=66). All cows that calved for the first time between February 2019 and January 2020 were eligible for enrollment. Cows were enrolled 8 weeks before calving and followed up during the first 6 months of lactation. In this study, we focused on milk samples collected during the first 35 DIM, and SCC and milk production from the first 6 months of lactation.

4.3.2 Milk sampling

Aseptic quarter milk samples were collected on a weekly basis during the first 5 weeks of lactation following procedures described by the National Mastitis Council (NMC, 2017). Briefly,

3 to 4 streams of milk were discarded after pre-dipping. Teat-ends were then thoroughly scrubbed with gauze squares soaked in 70% ethanol. Wearing clean gloves that were changed between each cow, quarter-level milk samples (approximately 10 mL) were collected in separate tubes. Samples were stored on ice immediately upon collection and frozen at -20 °C within 4 hours of collection. Quarter-level samples were later thawed and then pooled into a composite milk sample inside a laminar hood as previously described (Dean et al., 2022; Peña-Mosca et al., 2023b).

4.3.3. Milk culture

Using a cotton swab, the composite milk sample (approximately 100 μL) was plated onto Columbia CNA agar with 5% sheep blood and MacConkey agar. Agar plates were first incubated under aerobic conditions at 37°C for 18 to 24 hours. Plates were visually examined by a trained technician, in order to evaluate the presence of growth of morphologically distinct isolates. Milk samples were defined as contaminated when more than 3 distinct isolates were identified in the same composite milk sample (Dean et al., 2022). Following this, plates were re-incubated overnight for a total of 42-48 hours and re-examined. Taxonomic assignment of isolates harbored from non-contaminated composite milk samples was made using a Matrix-assisted laser desorption/ionization-time of flight (**MALDI-TOF**) mass spectrometer (**MS**) (MALDI Microflex LT Biotyper, Bruker Daltonics Inc.) as previously described (Randall et al., 2015; Jahan et al., 2021; Wieland et al., 2023). Isolates were re-cultured as needed to obtain individual colonies, and these distinct colonies were employed for identification. Briefly, the peak profiles of each isolate were compared to a reference spectra Biotyper reference library (Microflex version 7854; last updated on 02/19/2019). Following manufacturer's

recommendations, confidence scores were used in the following way: >2.0: species-level diagnosis; 1.8 to 2: genus-level diagnosis and <1.8: MALDI-TOF diagnosis not recorded and traditional identification methods used. An IMI was defined as a non-contaminated composite sample containing one or more colony forming units (e.g., 10 colony forming units/mL) of any cultured isolate.

4.3.4. Electronic farm records

From each farm, a copy of electronic farm records was acquired to retrieve information about monthly SCC measurements and milk production during the first 180 DIM. One farm from the 5 farms included in this study did not have a copy of electronic farm records; hence this farm was excluded from all analyses in this manuscript.

4.3.5. Data cleaning process

A flowchart describing the data cleaning process is presented in **Figure 4.1**. Five hundred three cows were initially enrolled but excluded for various reasons. Milk samples from cows with no calving date information (n=66), contaminated milk samples (n=162) or those with negative DIM (n=2) were excluded from the analysis. Because some cows were followed longer than expected, milk samples corresponding to a 6th postpartum sample or samples collected after 35 DIM (n=20) were also excluded from the analysis. In addition, observations from 125 cows were excluded due to missing monthly testing records. A total of 1,348 composite milk samples and 1,674 monthly tests from 333 cows were available for analysis.

4.4.6. Statistical analysis

All statistical analyses were performed using R version 4.3.2 (https://www.r-project.org/). The statistical code used for this study, including all relevant outputs, can be found online (https://fepenamosca.github.io/IMI-udder-health_performance-organic-dairies.io/). No priori sample size calculation was performed for this exploratory study. For analysis, microorganisms were grouped into different taxonomic groups: SAU; non-aureus *Staphylococcus* spp. and the closely related *Mammaliicoccal* species (**NASM**); *Strep* spp. and *Strep*-like organisms (**SSLO**) which include members from *Strep* genus, as well as *Strep*-like organisms (**SLO**) (e.g., *Enterococcus* spp., *Aerococcus* spp., *Lactococcus* spp. and *Micrococcus* spp.); gram negative bacteria; and "others" (i.e., microorganisms that did not belong to any of the previous groups). The period prevalence and persistence of IMI during the first 35 DIM were reported. Period prevalence was defined as the proportion of animals that had an IMI by a specific microorganism or taxonomic group in any of the composite milk samples submitted for milk culture. Persistent IMI was defined as harboring the same microorganism as reported by the laboratory for 2 or more samples after calving, regardless of whether the IMI occurred in consecutive samples or not. Transient IMI was defined as the presence of an IMI by a specific microorganism in only one milk sample in the first 35 DIM. For multivariable regression modeling, microorganisms with a low period prevalence (n<20) were excluded. This threshold was chosen because these microorganisms tended to be only present in a single farm or the analysis resulted in imprecise estimates. For the primary objective, the relationship between presence of IMI by a specific microorganism in any composite milk sample during the first 35 DIM and new high SCC (**NHSCC**; defined as presence of SCC>200,000 cells/mL for the first time in the first 6 months

of lactation) was investigated using Cox proportional hazards regression, with a robust sandwich estimator to account for the clustering of cows within farms, utilizing the "survival" package (Therneau et al., 2023). Cows were considered at risk from calving and followed up to 180 DIM. Cows were considered at risk from calving and followed up to 180 DIM. Cows were right-censored at the end of the follow-up period or when they were lost during follow-up, presumably due to culling, death, or unknown reasons. The proportional hazards assumption was tested using the Schoenfeld residuals. The association between presence of IMI by a specific microorganism in any composite milk sample in the first 35 DIM and milk production during the first 6 months of lactation was investigated using mixed linear regression ("nlme" package (Pinheiro et al., 2023)), with the inclusion of farm-ID and cow-ID (nested within farm) as a random effect, to account for non-independence of observations at each level. The correlation between repeated measurements within the same cow was further accounted for by the inclusion of an autoregressive correlation structure. All the models included an interaction term between presence of IMI and postpartum DHIA monthly test. Normality assumption was tested by a visual assessment of the model's residuals and the use of quantile-quantile plots. The relationship between IMI persistence by a specific microorganism (Persistent IMI, Transient IMI or no IMI by a given microorganism) and the number of monthly tests with high SCC during the first 180 DIM was investigated using negative binomial regression, as implemented in the "lme4" package (Bates et al., 2023). The dispersion of the data was investigated by use of the dispersion test (Cameron and Trivedi, 1990) and the comparison of model log-likelihood between negative binomial and Poisson regression models (Jackman et al., 2023). For negative binomial models, incidence rate ratios (IRR) were estimated by exponentiation of the coefficients from the models,

and Wald 95% confidence intervals were determined using the "Confint" function from the "car" package (Fox et al., 2023). Because cows had multiple IMI from different microorganisms during the first 35 DIM, the presence of IMI by other bacterial groups (e.g., SAU, NASM or SSLO) on the first postpartum sample was included as a confounder in all models when they were not the main explanatory variable in the model. When SAU-IMI was the main explanatory variable, confounders included in the models were the presence on the first postpartum sample of NASM-IMI and SSLO-IMI. For NASM-IMI, models accounted for the presence of SAU-IMI and SSLO-IMI on the first postpartum sample. In case of SLO, NASM-IMI and SAU-IMI on the first postpartum sample were accounted for in the models. For gram-negative-IMI and others-IMI (i.e., those not included in the previous groups), confounders included in the models were presence IMI of NASM and SAU or SSLO on the first postpartum sample. Results are presented and discussed taking into consideration the precision of the estimates (95% CI) instead of hypothesis testing (Poole, 2001; Greenland et al., 2016).

4.4. RESULTS

4.4.1 Descriptive characteristics

Descriptive data for the enrolled cows in each of the study farms are presented in **Table 4.1**. Enrolled farms were located in different regions of the United States including Texas (n=1), New Mexico (n=1), Colorado (n=1) and Minnesota (n=2). The herd size of enrolled farms ranged from 100 to 3,000 milking cows. All farms allowed access to pasture and cows consumed at least 30% of their DMI from pasture when possible following organic regulations. The housing systems differed between farms, with cows in Texas and New Mexico housed in dry lot

pens; cows in Colorado and one Minnesota farm in a free stall barn; and cows in the other Minnesota farm having access to a compost barn and out-wintering lot during the winter. The percentage of enrolled animals with a high SCC on the first postpartum DHIA test ranged from 31.4% and 82.3% across the four farms with DHIA data available; average milk production in these farms for enrolled cows ranged from 17.6 to 29.2 kg/d.

4.4.2. Culture results

Culture results during the first 35 DIM are presented in **Table 4.2**. During the first 35 DIM, 87.7% of the cows had an IMI in at least 1 composite milk sample submitted for milk culture. In this period, 21.9% of the cows (73/333) had a SAU-IMI, out which 18.9 % (63/333) were classified as persistent. Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species were isolated from 50.2 % (167/333) of the enrolled animals; 28.2% (96/333) of cows had persistent NASM-IMI. *Strep* spp. and *Strep*-like organisms were found in 36.5 % (121/333) of the cows in the present study, while 10.5% (35/333) of them had persistent SSLO-IMI. Gram-negative bacteria were found in 9.3% (31/333) of the enrolled cows; none of the gram-negative-IMI were persistent. Other microorganisms were found in 37.2% (124/333) of the enrolled animals.

4.4.3. Relationship between the presence of intramammary infections and hazards of new high SCC during the first 6 months of lactation

Figure 4.2 shows a summary of results from the Cox regression models assessing the association between presence of IMI during the first 5 weeks of lactation and NHSCC during the first 6 months of lactation. During the first 6 months of lactation, 53.5% (178/333) of the

enrolled cows had at least one test with high SCC. The presence of SAU-IMI (HR [95%CI]): 3.35 [2.64, 4.25]), NAS non-*chromogenes*-IMI (HR [95%CI]): 1.43 [1.06, 1.93]), and *Strep* spp.-IMI (HR [95%CI]: 2.25 [2.12, 2.39]) during the first 35 DIM was positively related to NHSCC during the first 6 months of lactation. For SCH-IMI the proportional hazards assumption was not met (Schoenfeld's residuals test $P < 0.001$) indicating the association between SCH-IMI and NHSCC varied over time. For SCH-IMI, there was no evidence of a difference in the hazards of NHSCC during the first 3 postpartum months after calving (HR [95%CI]: 0.87 [0.67, 1.12)]). However, a positive relationship with NHSCC was observed through the last 3 months of the follow-up period (HR [95%CI]: 2.25 [1.58, 3.19]). Gram-negative-IMI (HR [95%CI]: 1.14 [0.78, 1.67]) and SLO-IMI (HR [95%CI]: 1.08 [0.71-1.62]) showed no evidence of an association with NHSCC during the first 6 monthly tests. The presence of *Bacillus* spp.-IMI were associated with lower hazards of NHSCC during the first 6 months of lactation (HR [95%CI]: 0.45 [0.30, 0.68]). Hazards of NHSCC were numerically reduced in cows with *Corynebacterium* spp.,-IMI, although this reduction did not attain statistical significance (HR [95%CI]: 0.69 [0.38, 1,27]).

4.4.4. Relationship between presence of intramammary infections and milk production during the first 6 months of lactation

The average milk production (kg/day; mean (SD)) across all enrolled cows with available DHIA data was 24.8 (8.5), 27.3 (7.9), 26.5 (8.5), 26.2 (8.3), 25.2 (9.5) and 23.2 (9.3) in the 1st to 6th postpartum month, respectively. The presence of SAU-IMI in the first 35 DIM was not associated with milk production during the first 6 months of lactation (overall estimate [95%CI]: -0.005 [-1.32, 1.31]; **Figure 4.3A)**. *Staphylococcus chromogenes*-IMI was negatively associated with milk production in the 3rd monthly DHIA test (estimate [95%CI]: -1.8 [-0.2, -3.5] and

showed a numerical decrease in the 5th DHIA test (estimate [95%CI]: -1.4 [-3.1, 0.2]). However, we did not find evidence of a difference in milk production in other postpartum tests (**Figure 4.3B**). For NASM non-SCH, milk production was not significantly different in the first 5 postpartum months between cows with and without an IMI by these microorganisms; nonetheless presence of NASM-non-SCH-IMI was associated with increased milk production in the 6th postpartum month (estimate [95%CI]: 2.5 [0.4, 4.5]) (**Figure 4.3D and 4.3E**). *Streptococcus* spp.-IMI was associated with lower milk production during the first 6 months of lactation driven mostly by *Strep dysgalactiae* (the most prevalent IMI), the presence of which was associated with a reduction of milk production in the first 2 postpartum monthly tests (1st: estimate [95%CI]: -2.7 [-5.0, -0.4] and 2nd: estimate [95%CI]: -3.6 (-5.9, -1.3); **Figure 4.4B-D**). *Streptococcus*-like organisms (**Figure 4.4E-F**) and gram-negative-IMI (**Figure 4.5A**) were not associated with milk production during the lactation (estimate [95%CI] SLO: -0.2 [-1.7, 1.3]; GN: 0.01 [-1.8, 1.9]). *Bacillus* spp.-IMI was associated with higher milk production during the first 6 months of lactation (overall estimate [95%CI]: 1.7 [0.3, 3.0]; **Figure 4.5C**). Cows with *Corynebacterium* spp.-IMI had lower milk production in the first postpartum month (estimate [95%CI]: 1.7 [-3.9, 0.4], however, no evidence of a difference in milk production was observed thereafter; **Figure 4.5D**).

4.4.5. Relationship between persistence of intramammary infections and number of DHIA tests with high SCC during the lactation

Adjusted counts (±SE) from negative binomial models depicting the relationship between presence of transient or persistent IMI and number of monthly tests with high SCC are presented in **Figure 4.6**. During the first 6 months of lactation, 46.5% (156/333) of the enrolled cows did

not have any monthly DHIA test with high SCC, 21.6% (72/333) of the cows had 1 test with

high SCC, 7.8% (26/333) had 2 tests with high SCC and 24.0% (80/333) had 3 or more tests with

high SCC. The presence of persistent SAU-IMI was positively associated with the number of

tests with high SCC (IRR [95%CI]: 3.08 [2.31, 4.09]); while transient SAU-IMI was associated

with a small, but not statistically significant increase in the number of tests with high SCC (IRR

[95%CI]: 1.51 [0.81, 2.82]) compared to cows without SAU-IMI. Cows with transient and

persistent SCH-IMI had 1.23 (95%CI: 0.83, 1.82) and 1.17 (95%CI: 0.86, 1.59) times greater

number of tests with high SCC, respectively, when compared to cows without SCH-IMI.

However, is important to note that these differences were not statistically significant. Transient

NAS non-SCH-IMI showed no evidence of an increase in the number of tests with high SCC,

while for persistent NAS non-SCH-IMI, there was a significant increase compared to cows

without these microorganisms (IRR [95%CI]: 1.60 [1.00, 2.65]). Similarly, transient *Strep*-IMI

was associated with 1.27 times (95%CI: 0.87, 1.88) higher number of tests with high SCC, while

cows with persistent *Strep*-IMI showed 2.22 (95%CI: 1.52, 3.26) higher number of tests with

high SCC compared to cows without *Strep*-IMI. No evidence of an association was observed

between transient SLO-IMI and the number of tests with high SCC. The presence of persistent

SLO-IMI was related to a numerical increase in the number of tests with high SCC (IRR: 1.51

[95%CI]: 0.65, 3.53), and for persistent *Enterococcus* spp.-IMI (the most prevalent genus within

the SLO group in the present study) were related to 3.72 higher counts of high SCC in the

following lactation (IRR [95%CI]: 3.72 [1.35, 10.26]). Cows with gram-negative-IMI showed no

association with the number of tests with high SCC (IRR [95%CI]: 1.01 [0.61, 1.67]). Both

transient and persistent *Bacillus* spp.-IMI were related to lower number of tests with high SCC

during the lactation (IRR [95%CI]: 0.64 [0.44, 0.94]) and IRR [95%CI]: 0.15 [0.04, 0.66], respectively). Transient *Corynebacterium* spp.-IMI was not related to the number of DHIA tests with high SCC during the lactation (IRR [95%CI]: 0.96 [0.60, 1.54]); while persistent *Corynebacterium* spp.-IMI were related to a numerically lower, yet lacking statistical significance, decrease in the number of tests with high SCC (IRR [95%CI]: 0.54 [0.19, 1.54]).

4.5. DISCUSSION

This longitudinal study explored the relationship of the presence and persistence of IMI during the first 35 DIM with milk production and SCC during the first 6 months of lactation in primiparous organic dairy cows. This represents a relatively under investigated population of animals, in which restrictions placed on antibiotic usage in organic dairy farms, could increase the capability of mastitis-causing pathogens to persist within the mammary gland, therefore compromising the udder health of primiparous organic dairy cows. To our knowledge this is the first longitudinal study that investigated the relationship between presence and persistence of IMI, udder health and milk production in this population of dairy cows.

4.5.1. Relationship between presence of IMI, hazards of new high SCC and milk production during the lactation

In our study, we encountered a higher prevalence of SAU and *Strep* spp. than reported in prior investigations in conventional dairies. Nonetheless, the prevalence of IMI differed across farms in this study, a topic covered extensively in a preceding article (Peña-Mosca et al., 2023b). Interestingly, our observations revealed that certain bacterial microorganisms, especially SAU and *Staphylococcus chromogenes*, showed a high prevalence at calving, which suggests that

prepartum management should be a focus of IMI prevention and control in organically reared

primiparous cows. Lastly, some species showed a predominance of persistent-IMI (e.g., SAU,

Staphylococcus chromogenes and *Strep* spp.), while others caused primarily transient-IMI (e.g.,

NASM non-*chromogenes*, SLO, gram-negative microorganisms) (Peña-Mosca et al., 2023b).

Numerous factors could potentially explain the difference in IMI prevalence across organic and

conventional dairy farms. These factors include not only those related to the limitations imposed

on antimicrobial usage in organically managed dairy farms but also variations in herd sizes,

utilization of farm records, participation in DHIA, and availability of veterinary support

(Stiglbauer et al., 2013).

In our study, we discovered that presence of IMI caused by gram-positive

microorganisms (SAU, *Strep* spp.) was associated with an increase in the hazards of NHSCC

during the lactation. This finding agrees with reports from conventional dairy farms, and

conforms to the well-documented ability of bacteria from these genera to cause subclinical and

clinical mastitis during the lactation (Keane, 2019). Unexpectedly, we did not find evidence of a

negative association between the presence of SAU-IMI and milk production during the lactation.

This is surprising given the wide variety of virulence factors and toxins that this microorganism

possesses (Rainard et al., 2018; Fergestad et al., 2021). However, previous studies performed in

conventionally managed heifers have also reported the lack of a statistically significant

association between SAU-IMI and milk production (Paradis et al., 2010). One potential factor

that could explain these results is the fact that cows with higher milk production have a higher

susceptibility to SAU-IMI (Gröhn et al., 2004), therefore biasing the results towards the null.

Still, within the scope of our study, the importance of this factor might be diminished, as the

majority (76.6%) of SAU-IMI cases were already evident on the first composite milk sample after calving (Peña-Mosca et al., 2023b). *Streptococcus* spp.-IMI were associated with lower milk production in the following lactation, which concurs with previous reports (Piepers et al., 2010; Valckenier et al., 2020) and indicates the potential importance of control of these bacteria in organically reared animals.

Presence of NASM in milk samples was associated with increased hazards of NHSCC during the lactation, although this association varied by both species (i.e., SCH vs NASM non-SCH) and DIM (<90 vs >90 DIM). In addition, our findings suggested that the relationship between presence of NASM-IMI in the first 35 DIM and milk production during the first 6 months of lactation varied by the different species, in which SCH-IMI and other NASM were negatively and positively associated with milk production in one or more postpartum tests, respectively. Non-*aureus Staphylococcus* and closely related *Mammaliicoccal* species have been hypothesized as potential candidates for the development of probiotics to control mastitis (De Buck et al., 2021) due to their protective ability against major pathogens (Reyher et al., 2012) and positive relationship with milk production in some studies (Piepers et al., 2010). Our results contradict this hypothesis. One reason for this discrepancy could be differences in inhibitory activity between different species or strains of NASM (De Buck et al., 2021). Indeed, prior research has shown that certain NASM species are related to higher SCC and lower milk production, including SCH, *Staphylococcus xylosus* and *Staphylococcus simulans* (Supré et al., 2011). Except for SCH (386/512, 75.4%), we could not explore this relationship with NASM species isolated in this study because they showed a low prevalence and/or could not be speciated using MALDI-TOF MS (110/126, 87.3%). To overcome this limitation, future research

may look into alternative cut-off points for identifying NASM species (Cameron et al., 2017) and

the use of extended databases with NASM bovine isolates (Cameron et al., 2018) as potential

alternatives to improve the limited taxonomic resolution of MALDI-TOF MS on NASM when

manufacturer's databases and recommendations are followed.

In our study, gram-negative-IMI in early lactation were not associated with NHSCC or

milk production during the first 180 DIM. Gram-negative bacteria are an important cause of

clinical mastitis in conventionally managed farms (Klaas and Zadoks, 2018); however, most

species belonging to this group are poorly adapted to the mammary gland and typically result in

transient IMI (Klaas and Zadoks, 2018; Peña-Mosca et al., 2023b). This could potentially explain

the lack of long-term relationships between gram-negative-IMI and udder health and milk

production during the lactation. Isolation of *Bacillus* spp. was related to half the hazards of high

SCC during the lactation and increased milk production throughout the lactation. This

microorganism is classified as an environmental and opportunistic gram-positive mastitis

pathogen and is poorly described in the literature. *In vitro* studies have shown promising results

regarding *Bacillus*' ability to modulate and inhibit the growth of major mastitis pathogens such

as SAU (Al-Qumber and Tagg, 2006; Gutiérrez-Chávez et al., 2016; Afroj et al., 2021; Qiu et al.,

2022). This inhibitory activity could perhaps explain the beneficial relationships between

positive growth of *Bacillus* in milk culture and high SCC and milk production during lactation.

Given the relative novelty of this observation, this hypothesis requires further investigation.

Corynebacterium is another bacterial genus with potential protective activity in the mammary

gland (Reyher et al., 2012). In this longitudinal study, cows with *Corynebacterium*-IMI had

lower hazards of NHSCC and higher milk output in the first postpartum month, although the

estimates related to high SCC hazards were relatively imprecise and this association was not statistically significant.

4.5.2. Relationship between presence and persistence of IMI and number of tests with high SCC

Our third objective was to investigate if the IMI persistence was associated with the number of DHIA tests with high SCC during the lactation. For the description of the epidemiology of mastitis microorganisms, pathogens are frequently grouped into categories based on whether they are well-adapted to the mammary gland and have cow-to-cow transmission (i.e., contagious) or poorly adapted to the mammary gland and commonly found in the environment (Zadoks et al., 2011; Keane, 2019). The epidemiology of mastitis microorganisms has large implications for decision-making, such as antibiotic therapy in dairy farms using on-farm culture results (Lago and Godden, 2018). Epidemiological knowledge of these pathogens can also be important for prognosis of quarters infected by host-adapted pathogens, since infections tend to persist for long periods if they remain untreated (Barkema et al., 2006; Bardiau et al., 2014), as is the case of organically managed dairy cows (NMC, 2019).

In our study, a relatively large proportion of the IMI were determined to be persistent, but this varied across different microorganisms. Specifically, SSLO and SAU exhibited a moderate-to-high persistence in the mammary gland, and animals with persistent IMI with these groups of bacteria had a much larger increase in the number of tests with high SCC compared to the increase seen in transiently infected animals. The differential relationship between transient versus persistent IMI and udder health could have implications for the understanding of the relationship between IMI and udder health; and suggests that persistent IMI during the first 35

DIM have a strong impact on SCC during the first 180 DIM. In addition, host-adapted microorganisms tend to have a contagious epidemiology (Keane, 2019), representing an additional threat for non-infected cows if they are managed and milked together with infected animals (Dufour et al., 2012). The presence of transient or persistent NASM-IMI was related to a mild increase in the number of DHIA tests with high SCC compared to cows without NASM-IMI. This agrees with the former classification of NASM as minor pathogens (De Buck et al., 2021); and contradicts previous studies that found that these microorganisms have a protective effect on udder health (Piepers et al., 2010; Reyher et al., 2012). Conversely, based on our results, presence of transient and especially persistent *Bacillus*-IMI was related to a significant reduction in the number of tests with high SCC throughout the lactation. The observed protective effect of this bacterial genus (in terms of both milk production and SCC) highlights its potential opportunity for prevention and control of mastitis in organically reared heifers. This hypothesis should be confirmed in future studies, as discussed above.

4.5.3. Validity

This study included data from 4 organic dairy farms with variable herd sizes and located in different regions of the United States, which increases external validity from this study.

Limitations of this study include the risk of potential confounding because unmeasured or unknown confounders could be biasing the results. For instance, genetic potential for milk production (Gröhn et al., 2004; Piepers et al., 2013; Nitz et al., 2020), as well as, other known risk factors for IMI such as udder edema, milk leakage, high or low body condition around calving and age at first calving (Nitz et al., 2020; Fernandes et al., 2022). Therefore, the results from this exploratory study should be interpreted with caution.

Additionally, selection bias could represent an additional source of systematic error, due to the exclusion of observations with missing data, especially considering the high proportion of cows in our study with missing DHIA data (27.3% [125/458]) (Hernán et al., 2004). Twenty-one of these cows were missing because of missing DHIA testing in farm D. However, the remaining 104 animals for other unknown reasons.

The use of composite milk samples represents a potential limitation of this observational study, considering the lower sensitivity of milk culture to detect IMI (Reyher and Dohoo, 2011; Toft et al., 2019). Nonetheless, this limitation was overcome using repeated sampling over the first 35 DIM. A method that improves the sensitivity of milk culture for detection of IMI (Buelow et al., 1996).

Because we evaluated the presence of IMI in the first 35 DIM and the milk production and SCC during the first 180 DIM (including the first 35 DIM), it is possible that an IMI was detected following or simultaneously with the first DHIA test in where the outcome was evaluated for the first time. This sequence of events could introduce challenges in establishing a temporal relationship between the studied exposures and outcomes and might even result in reverse causality concerns. To address this problem, and considering that having an IMI by one microorganism might directly influence the likelihood of the presence of other microorganisms (e.g., through bacteriocin production) or indirectly affect it (e.g., by elevating SCC) (Reyher et al., 2012), in all models we also accounted for presence of IMI on the first postpartum week by other microorganisms of importance for udder health as a potential confounder.

In this study we did not take into account consecutiveness for defining IMI persistence (Peña-Mosca et al., 2023b). We opted this approach due to the lower sensitivity of composite

milk samples in detecting IMI within a single milk sample (Dohoo et al., 2011; Toft et al., 2019). Due to this, we held the belief that within the repeated samples, the presence of one negative result does not necessarily indicate the absence of IMI. Besides, when we compared IMI persistence definitions, we discovered the vast majority of IMI defined as persistent were the result of consecutive persistent IMI (e.g., SAU: 85.7% [54/63], *Staphylococcus chromogenes*: 90.0% [72/80] and for *Streptococcus dysgalactiae*: 86.4% [19/22]). While offering an improvement over less accurate methods, MALDI-TOF MS still possesses limitations for the assessment of IMI persistence, particularly in cases where isolates are only able to be identified with confidence at the genus level (Jahan et al., 2021).This also does not take into account the possibility of different strains infecting the animal on successive samples. Our findings might therefore be overestimating IMI persistence in these circumstances. In addition, it is known that the persistence of IMI can differ depending on the immune system's capacity to combat mastitis pathogens (Doymaz et al., 1988; Leelahapongsathon et al., 2020). This capability is compromised during early lactation (Sordillo, 2018), period in which IMI persistence was evaluated in this study. Consequently, these factors have the potential to alter the association between IMI persistence and udder health in the present study.

This study lacked a priori sample size calculation, and it was not specifically designed to confirm these hypotheses. Instead, it was intended as an exploratory investigation of the associations between the presence and persistence of IMI by various microorganisms on udder health and milk production. Therefore, more research is necessary to confirm these findings and to gain a more comprehensive understanding of the relationship between IMI and udder health in first-lactation organic dairy cows.

4.6. CONCLUSIONS

The presence of IMI by SAU or *Strep* spp. in the first 35 DIM was associated with an increased likelihood of NHSCC during the first 6 months of lactation. Milk production during the first 180 DIM was lower when *Strep* spp. was identified in milk samples, however, it was not different in cows with SAU-IMI. Isolation of NASM was associated with a mild increase in the hazards of NHSCC and changes in milk production that varied across the different microorganisms within this group. The presence of gram-negative- or SLO-IMI in the first 35 DIM was not related to NHSCC or milk production during the first 180 DIM. The presence of *Bacillus*-IMI in the first 35 DIM was related to lower hazards of NHSCC, reduced number of tests with high SCC and higher milk production during the first 180 DIM. The persistence of IMI in the first 35 DIM was associated to the number of monthly DHIA tests with high SCC during the lactation for all microorganisms except for SCH. Despite the apparent differences in the dynamics and distribution of mastitis pathogens in this study, in comparison to previous reports from conventional dairy cows, our study showed associations between IMI, udder health, and milk production that are align with findings from conventionally managed first-lactation cows. Based on our findings, SAU and *Strep* spp. exhibited moderate to high persistence in the mammary gland, associated with a substantial increase in the risk of NHSCC. Therefore, special care should be taken in management and prevention strategies to address these highly prevalent and significant mastitis pathogens in first-lactation organic cows.

4.7. ACKNOWLEDGMENTS

4.9. TABLES AND FIGURES

4.9.1 Tables

Table 4.1. Descriptive characteristics of enrolled animals (n=354).

Farm	n	Location	Herd size	Housing system	Prevalence any IMI	Prevalence high SCC first test	Milk production (Kg/day) first test
A	49	Colorado	1700	Free stall barn with exercise pen	46.9% (23)	51.1% (24)	22.5 (5.13)
B	116	Texas	3000	Dry lot	91.4% (106)	59.4% (57)	29.2 (8.87)
C	105	New Mexico	1500	Dry lot	95.2% (100)	31.4% (33)	26.1 (7.27)
D †	21	Minnesota	100	Free stall barn	95.2% (20)	Not available	Not available
E	63	Minnesota	300	Compost barn and out-wintering lot during winter	100% (63)	82.3% (51)	17.6 (7.25)

† Because farm D (n=21) did not have DHIA data available it was excluded from the analysis.
SCC: somatic cell count. IMI: intramammary infections.

Table 4.2. Proportion and number of cows that had an intramammary infection (IMI) or a persistent IMI during the first 5 weeks of lactation (n=333)

Taxonomic group	% IMI (n)	% Persistent IMI (n)
Any IMI	87.7% (292)	-
Staphylococcus aureus	21.9% (73)	18.9% (63)
NASM	50.2% (167)	28.2% (96)
Staphylococcus chromogenes	36.3% (121)	24.0% (80)
NASM non-*chromogenes*	21.9% (73)	6.6% (22)
Staphylococcus haemolyticus	1.8% (6)	0% (0)
Staphylococcus hominis	0.3% (1)	0% (0)
Staphylococcus sciuri	1.2% (4)	0% (0)
Staphylococcus xylosus/*saprophyticus*	0.3% (1)	0% (0)
Unspeciated *Staphylococcus*	19.5% (65)	6.6% (22)
SSLO	36.5% (121)	10.5% (35)
Streptococcus spp.	20.1% (67)	9.3% (31)
Streptococcus dysgalactiae	12.9% (43)	6.6% (22)
Streptococcus uberis	2.7% (9)	1.2% (4)
Unspeciated *Streptococcus*	9.3% (31)	2.7% (9)
Streptococcus-like organisms	18.0% (60)	2.4% (8)
Aerococcus spp.	4.2% (14)	0% (0)
Aerococcus viridans	3.3% (11)	0% (0)
Unspeciated *Aerococcus*	1.2% (4)	0% (0)
Enterococcus spp.	11.7% (39)	1.2% (4)
Enterococcus casseliflavus	3.6% (12)	0.3% (1)
Enterococcus hyrae	2.4% (8)	0% (0)
Enterococcus mundtii	3.0% (10)	0% (0)
Enterococcus faecalis	0.3% (1)	0% (0)
Enterococcus saccharolyticus	0.3% (1)	0% (0)
Unspeciated *Enterococcus*	4.2% (14)	0.9% (3)
Lactococcus spp.	1.5% (5)	0% (0)
Lactococcus garvieae	0.9% (1)	0% (0)
Lactococcus lactis	0.4% (1)	0% (0)
Unspeciated *Lactococcus*	0.3% (1)	0% (0)
Micrococcus spp.	1.2% (4)	0% (0)
Gram-negative	9.3% (31)	0% (0)
Escherichia spp.	3.0% (10)	0% (0)
Escherichia coli	3.0% (10)	0% (0)
Unspeciated *Escherichia*	0.6% (2)	0% (0)
Klebsiella oxytoca	0.9% (3)	0% (0)
Pseudomonas spp.	0.9% (3)	0% (0)
Citrobacter freundii	0.3% (1)	0% (0)
Enterobacter spp.	0.6% (2)	0% (0)
Enterobacter cloacae	0.3% (1)	0% (0)
Unspeciated *Enterobacter*	0.3% (1)	0% (0)
Pantoea spp.	0.9% (3)	0% (0)
Serratia spp.	0.9% (3)	0% (0)
Unidentified gram-negative	1.2% (4)	0% (0)
Other microorganisms	37.2% (124)	9.3% (31)
Arthrobacter gandavensis	0.6% (2)	0% (0)
Unspeciated *Arthrobacter*	0.3% (1)	0% (0)
Bacillus spp.	23.7% (79)	5.4% (18)
Corynebacterium spp.	15.9% (53)	3.6% (12)
Helcococcus ovis	0.3% (1)	0% (0)
Trueperella pyogenes	0.6% (2)	0% (0)
Unidentified gram-positive cocci	4.2% (14)	0% (0)
Unidentified gram-positive rod	0.3% (1)	0% (0)
Yeast	0.6% (2)	0% (0)

NASM: non-aureus *Staphylococci* and closely related *Mammaliicoccal* species. SSLO: *Streptococcus* spp. and *Streptococcus*-like organisms.

4.9.2 Figures
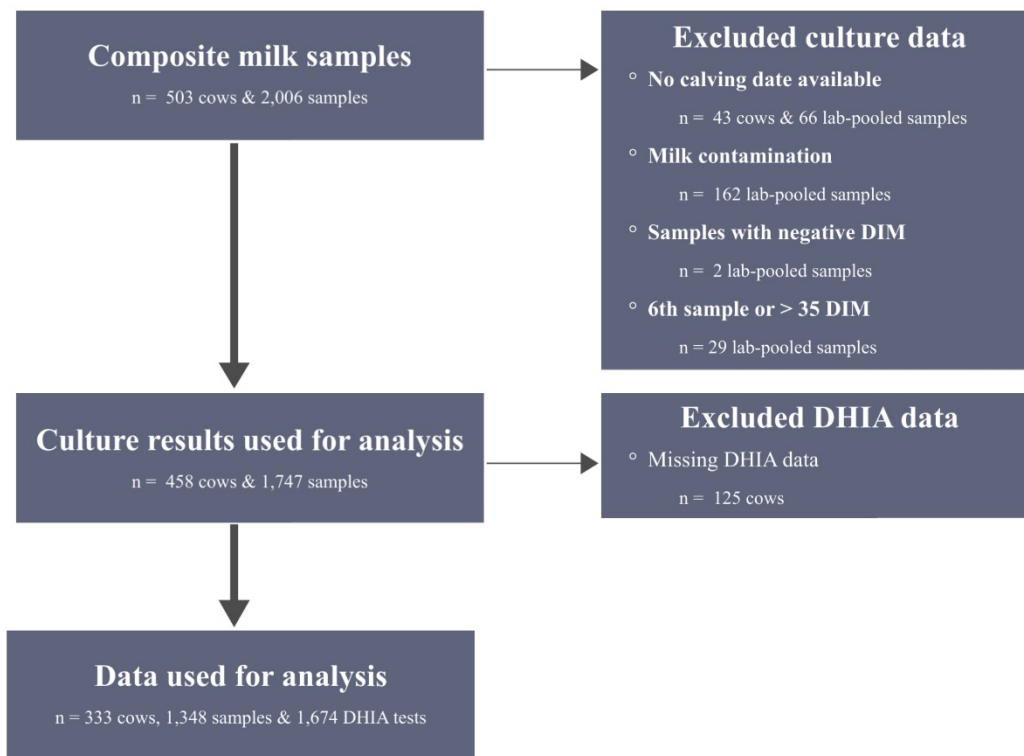
Figure 4.1. Flowchart describing observations excluded during data cleaning process. On the left side, the number of cows and samples at each stage of the data cleaning process is illustrated. On the right side, the number of cows and samples that were excluded, along with the associated reasons for exclusion, are shown.

Taxonomic group	Crude risk exposed	Crude risk unexposed		HR (95% CI)
Staphylococcus aureus	84.9% (62/73)	44.6% (116/260)		3.35 (2.64 to 4.25)
NASM	58.7% (98/167)	48.2% (80/166)		1.34 (0.97 to 1.85)
Staphylococcus chromogenes: <90 DIM	45.5% (55/121)	41.5% (88/212)		0.87 (0.67 to 1.12)
Staphylococcus chromogenes: >90 DIM	60.3% (73/121)	49.5% (105/212)		2.25 (1.58 to 3.19)
NASM non-chromogenes	60.3% (44/73)	51.5% (134/260)		1.43 (1.06 to 1.93)
Unspeciated Staphylococcus	60.0% (39/65)	51.9% (139/268)		1.38 (1.03 to 1.84)
SSLO	64.5% (78/121)	47.2% (100/212)		1.86 (1.74 to 1.99)
Streptococcus spp.	79.1% (53/67)	47.0% (125/266)		2.25 (2.12 to 2.39)
Streptococcus dysgalactiae	81.4% (35/43)	49.3% (143/290)		2.08 (1.72 to 2.51)
Unspeciated Streptococcus	77.4% (24/31)	51.0% (154/302)		1.76 (1.26 to 2.48)
Streptococcus-like organisms	46.7% (28/60)	54.9% (150/273)		1.08 (0.71 to 1.62)
Enterococcus spp.	43.6% (17/39)	54.8% (161/294)		1.02 (0.55 to 1.92)
Gram-negative	51.6% (16/31)	53.6% (162/302)		1.14 (0.78 to 1.67)
Others	38.7% (48/124)	62.2% (130/209)		0.55 (0.35 to 0.88)
Bacillus spp.	30.4% (24/79)	60.6% (154/254)		0.45 (0.30 to 0.68)
Corynebacterium spp.	41.5% (22/53)	55.7% (156/280)		0.69 (0.38 to 1.27)

0.5 1 2 3 4

← Lower hazards high SCC   Higher hazards high SCC →

Figure 4.2. Summary of Cox regression models investigating the relationship between intramammary infections (IMI) by a specific microorganism during the first 35 days in milk and new high somatic cell count (SCC) (SCC>200,000 cells/mL) during the first 180 days in milk. Only microorganisms present in at least 20 animals were analyzed. NASM: non-*aureus Staphylococci* and closely related *Mammaliicoccal* species. SSLO: *Streptococcus* spp. and *Streptococcus*-like organisms. When *Staphylococcus aureus* (SAU)-IMI was the main explanatory variable, confounders included in the models were the presence on the first postpartum sample of NASM-IMI and SSLO-IMI. For NASM-IMI, models accounted for the presence of SAU-IMI and SSLO-IMI on the first postpartum sample. In case of *Streptococcus*-like organisms, NASM-IMI and SAU-IMI on the first postpartum sample were accounted for in the models. For gram-negative-IMI and others-IMI (i.e., those not included in the aforementioned groups), confounders included in the models were presence IMI of NASM and SAU or SSLO on the first postpartum sample.
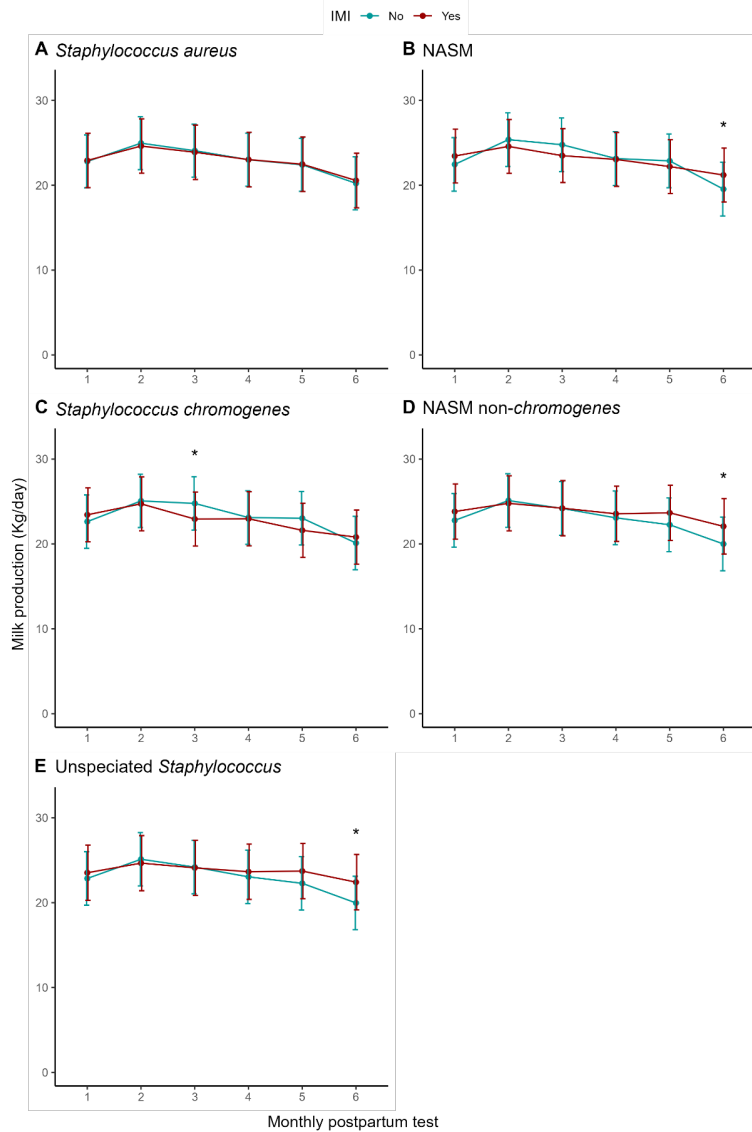
Figure 4.3. Milk production (kg/day) during the first 180 days in milk for cows with and without a *Staphylococcus* spp. intramammary infections (IMI) during the first 35 days in milk. Error bars represent standard errors. Asterisks indicate statistically significant difference between both groups within a given time point (*P* <0.05). Only microorganisms present in at least 20 animals were analyzed. NASM: non-*aureus Staphylococci* and closely related *Mammaliicoccal* species. For *Staphylococcus aureus*, models controlled for presence of NASM-IMI and *Streptococcus* spp. and *Streptococcus*-like organisms-IMI on the first postpartum sample. For NASM, models controlled for presence of *Staphylococcus aureus*-IMI and *Streptococcus* spp. and *Streptococcus*-like organisms-IMI on the first postpartum sample.
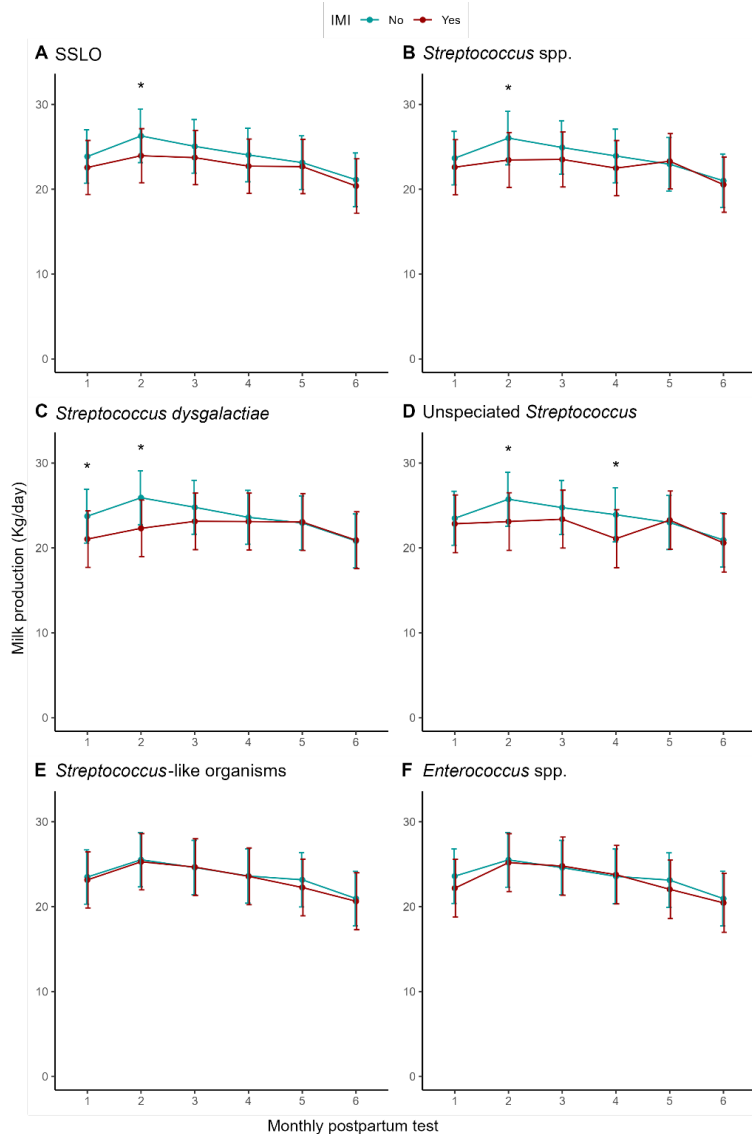
Figure 4.4. Milk production (kg/day) during the first 180 days in milk for cows with and without a *Streptococcus* spp. and *Streptococcus*-like organisms (SSLO) intramammary infections (IMI) during the first 5 weeks of lactation. Error bars represent standard errors. Asterisks indicate statistically significant difference between both groups within a given time point (P <0.05). Only microorganisms present in at least 20 animals were analyzed. Confounders included in the models were presence of non-aureus *Staphylococci* and closely related *Mammaliicoccal* species-IMI and *Staphylococcus aureus*-IMI on the first postpartum sample.
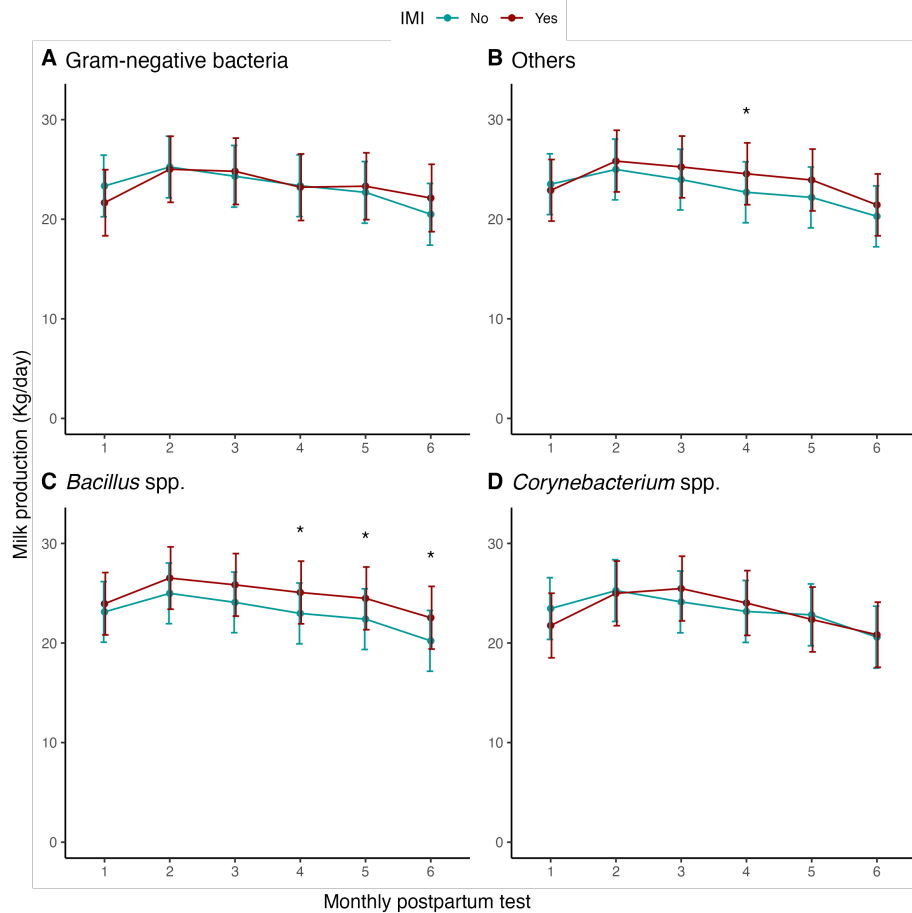
Figure 4.5. Milk production (kg/day) during the first 180 days in milk for cows with and without an intramammary infection (IMI) caused by gram-negative bacteria or other microorganisms during the first 5 weeks of lactation. Error bars represent standard errors. Asterisks indicate statistically significant difference between both groups within a given time point (P <0.05). Only microorganisms present in at least 20 animals were analyzed. Confounders included in the models were presence of non-aureus *Staphylococci* and closely related *Mammaliicoccal* species-IMI and *Staphylococcus aureus*-IMI or *Streptococcus* spp. and *Streptococcus*-like organisms - IMI on the first postpartum sample.
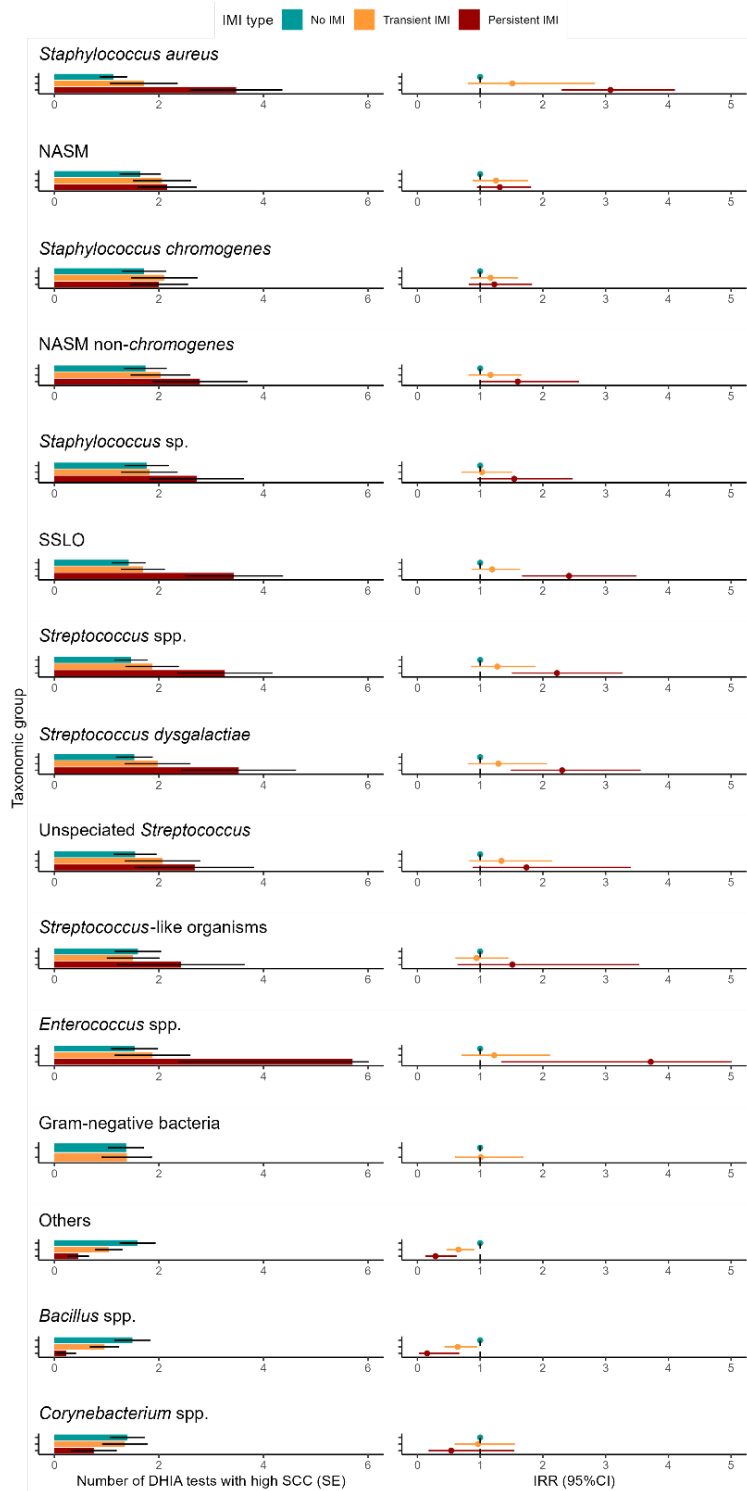
Figure 4.6. Relationship between transient and persistent intramammary infections (IMI) by a specific microorganism during the first 5 weeks of lactation and number of DHIA tests with high

somatic cell count (SCC) (SCC>200.000 cells/mL) during the first 6 monthly tests. Only microorganisms present in at least 20 animals were analyzed. When *Staphylococcus aureus* (SAU)-IMI was the main explanatory variable, confounders included in the models were the presence on the first postpartum sample of non-aureus *Staphylococci* and closely related *Mammaliicoccal* species (NASM)-IMI and *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**)-IMI. For NASM-IMI, models accounted for the presence of SAU-IMI and SSLO-IMI on the first postpartum sample. In case of *Streptococcus*-like organisms, NASM-IMI and SAU-IMI on the first postpartum sample were accounted for in the models. For gram-negative-IMI and others-IMI (i.e., those not included in the aforementioned groups), confounders included in the models were presence IMI of NASM and SAU or SSLO on the first postpartum sample.

CHAPTER 5: IN VITRO ANTIMICROBIAL ACTIVITY OF NON-AUREUS STAPHYLOCOCCI AND CLOSELY RELATED MAMMALIICOCCAL SPECIES AGAINST STAPHYLOCOCCUS AUREUS AND STREPTOCOCCUS UBERIS AND THEIR RELATIONSHIP WITH THE PRESENCE OF INTRAMAMMARY INFECTIONS IN ORGANIC DAIRY COWS

5.1. SUMMARY

Organic dairy farms are in need of novel strategies to control mastitis. Non-*aureus* *Staphylococci* and closely related *Mammaliicoccal* species (**NASM**) have been proposed as potential targets for the development of products to control mastitis, but their protective activity against major mastitis pathogens requires further investigation. This study aimed to: 1) Assess the *in vitro* antimicrobial activity of NASM from organic cow teat apices with and without intramammary infections (**IMI**) by *Staphylococcus aureus* (**SAU**) or *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**). 2) Investigate the relationship between IMI status and NASM counts. 3) Explore the relationship between NASM species assigned taxonomy and the inhibitory activity against SAU and *Streptococcus uberis* (**SUB**). A total of 114 cows in 2 farms were sampled and followed from 8 weeks before calving to 5 weeks after calving. Quarter-level milk samples were collected aseptically on a weekly basis during the first 5 weeks of lactation. These samples were then pooled into composite samples and submitted for milk culture. Milk culture results were used for the design of a case control study. Cases were defined as cows that had an IMI caused by SAU or SSLO during the first 35 days in milk (**DIM**). Controls were randomly chosen from cows that did not have an IMI caused by these microorganisms during the first 35 DIM. Cases and controls were matched by farm, lactation number, and DIM of IMI diagnosis (±7 DIM). From each selected cow, one teat apex sample from the week prior to IMI diagnosis was aerobically cultured. Taxonomy was confirmed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass spectrometry and whole genome sequencing. The inhibitory activity, including the minimum inhibitory concentration (MIC; $\log_{10}$, colony forming units/mL) of NASM isolates against SAU and SUB was investigated using agar dilution

methods. Isolates were ranked according to their *in vitro* inhibitory activity against SAU and SUB. Cox proportional hazards regression was used to assess the association between NASM taxonomy and NASM *in vitro* growth inhibition on SUB and SAU. The NASM MIC against SAU and SUB was further explored and compared across cases and controls as well as different NASM species using mixed linear regression. The relationship between IMI status and the presence of a "top 10" NASM isolates on the teat apex was investigated using mixed logistic regression. Fisher exact was used to compare the presence of isolates classified as "top 10" across different NASM species. The presence of NASM isolates classified as "top 10" with the lowest MIC against SAU was highest in controls (19.4%) compared to cases (5.8%). In this study, we found no evidence of an association between NASM counts on teat apex between cases and controls. The inhibitory activity of NASM was different across different NASM species. This study provides initial information about the inhibitory capabilities of naturally occurring NASM isolated against mastitis pathogens and its relationship with udder health in organic dairy cows.

5.2. INTRODUCTION

Mastitis is the leading cause of morbidity (USDA, 2007) and milk production loss in dairy farms (Heikkila et al., 2018). Although preventive measures are important for the control of mastitis, antimicrobials are still a major component of on-farm contagious mastitis control (Ruegg, 2017). However, the availability of antimicrobials for dairy farmers and veterinarians is at-risk due to concerns about antimicrobial resistance to medically important antibiotics and policy restrictions on antimicrobial use in livestock (Rajala-Schultz et al., 2021; McCubbin et al.,

2022; Ruegg, 2022b). This is especially true on organic-certified dairy farms, where limitations on antimicrobial usage are imposed (NMC, 2019).

Prior data from our group showed that *Staphylococcus aureus* (**SAU**) and *Streptococcus* spp. had a high prevalence and persistence in the mammary gland of dairy cows in organic farms in the first 35 days of lactation (Peña-Mosca et al., 2023b). Moreover, this study showed that SAU and *Streptococcus* spp. in early lactation were associated with a higher risk of high somatic cell count (**SCC**) during the first 180 days of lactation in organic dairy cows (Peña-Mosca et al., 2023a). Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (**NASM**) are also highly prevalent in milk samples (De Buck et al., 2021; Peña-Mosca et al., 2023b), but unlike SAU and *Streptococcus*, NASM have been associated with increased milk production (Piepers et al., 2010). Indeed, the presence of NASM has been associated with decreased risk for intramammary infection (**IMI**) with SAU and *Streptococcus* spp., suggesting a potential protective effect of NASM against these two pathogens (Reyher et al., 2012). *In vitro* studies using isolates obtained from the mammary gland of dairy cows have shown that NASM are capable of inhibiting the growth of SAU (De Vliegher et al., 2004b; Carson et al., 2017; Toledo-Silva et al., 2022). Using the cross-streak method for inhibitory testing, these studies reported that between 9.1% and 14.3% of the isolates were able to inhibit the growth of SAU (Carson et al., 2017; Toledo-Silva et al., 2022). Recently, agar dilution methods have also been utilized to investigate the inhibitory capabilities of NASM species on mastitis pathogens (Toledo-Silva et al., 2022). Moreover, studies in humans have also reported that specific NASM strains were associated with a decreased risk of skin colonization by SAU (Nakatsuji et al., 2017). These findings have raised the hypothesis that certain NASM species could be used for the

development of products to prevent or treat mastitis in dairy farms (De Buck et al., 2021).

However, despite the *in vitro* antimicrobial activity demonstrated by NASM, it remains uncertain

whether any species or strains within the NASM group would yield *in vivo* protective effects

against IMI by major mastitis pathogens. Lastly, the use of the agar dilution method has only

been investigated using a few isolates, and a more comprehensive investigation needs to be

performed to assess the inhibitory activity of different NASM species.

The first objective of this study was to investigate the phenotypic *in vitro* activity of

NASM isolated from the teat apex of organic cows with and without an IMI caused by SAU or

Streptococcus spp. and *Streptococcus*-like organisms (**SSLO**) on the week preceding IMI

diagnosis. We hypothesized that NASM isolates from cows without an IMI caused by SAU or

SSLO (i.e., controls) would exhibit higher *in vitro* inhibitory activity against SAU and

Streptococcus uberis (**SUB**) compared to NASM isolates from cows with SAU or SSLO IMI

(i.e., cases). Our second objective was to investigate the relationship between the presence of an

IMI caused by SAU or SSLO and NASM counts on teat-apex swab samples collected the week

prior to the diagnosis of SAU or SSLO IMI. We hypothesized that the NASM counts from the

teat apex samples would be higher in organic cows without an IMI compared to cows with an

IMI. Lastly, our third objective was to explore the relationship between the assigned taxonomy

of NASM species and the presence of inhibitory activity against SAU and SUB. We

hypothesized that there will be differences in the *in vitro* inhibitory across different NASM

species, with a few specific species showing high *in vitro* antimicrobial activity.

5.3. MATERIALS AND METHODS

The Strengthening the Reporting of Observational Studies in Epidemiology - Veterinary (**STROBE-Vet**) Statement guidelines were followed for the preparation of this manuscript (Sargeant et al., 2016). All study activities were approved by the University of Minnesota Institutional Animal Care and Use Committee (**IACUC**) (Protocol Number: 1807: 36109A), Colorado State University IACUC (Protocol number: 1442), and Texas Tech University IACUC (Protocol number: 18068-10). Laboratory activities were approved by the Institutional Biosafety Committee (IBC) protocol: 2205-40001H.

5.3.1 Inclusion criteria

Only cows from USDA organic certified dairy farms were enrolled in this study. Farms were selected from different regions of the United States and with different herd sizes. Enrollment was based on willingness to participate in the study, and proximity to the Universities involved in the study. During the enrollment process, we attempted to include farms that possessed electronic farm records. However, in order to maintain a balanced representation of both small and large dairy farms in the study, we ultimately included a small farm even though it lacked digital farm records. This study was part of a larger research initiative to investigate potential associations between the udder microbiome and udder health (Dean et al., 2021). For this purpose, cows from 5 organic dairy farms were enrolled 8 weeks before calving and followed up during the first 5 weeks of lactation (Peña-Mosca et al., 2023b). For this manuscript, we included data from only two of these farms because teat apex samples for bacteriological culture were collected in only these two farms due to limited research labor availability. Specifically, we used samples collected from 114 cows (Farm A [n =93] and Farm B

[n =21]). Farm A samples were collected between August 2019 and January of 2020 and Farm B samples were collected between February and July of 2021.

5.3.2 Implementation of case control study

Cases were defined as cows that had an IMI caused by SAU or SSLO during the first 35 days in milk (**DIM**). Controls were randomly chosen from the list of enrolled cows at Farms A and B that did not have an IMI caused by these microorganisms during the first 35 DIM. Importantly, it should be emphasized that milk samples collected from both cases and controls might have contained an IMI caused by other microorganisms (e.g., gram-negative bacteria, NASM, etc). However, these infections were not taken into account during the process of selecting cases and controls or in the subsequent analysis. Cases and controls were matched by farm, lactation number, and DIM of when SAU or SSLO IMI were diagnosed (±7 DIM). From each selected cow, one teat apex sample from the week prior to the diagnosis of a SAU or SSLO IMI was processed using the protocol outlined below. Because 3 cows did not have a teat apex sample available in the week prior to first diagnosis of SAU or SSLO IMI, 2 samples were processed for these animals, resulting in a total of 71 samples from 68 cows (34 cases and 34 controls).

5.3.3. Teat apex sample collection

All enrolled cows were sampled weekly from 8 weeks before calving to 5 weeks after calving. First, teats were visually inspected, and any visible debris was removed using a paper towel. Then, using a new pair of disposable gloves, a single sterile pre-moistened gauze was used to thoroughly swab the distal third of all four teats. After sampling, gauze was placed in

individual tubes containing a pre-loaded solution of 50% glycerol. Gauze samples were transported in a cooler with ice and stored in -80 °C after arrival to research facilities. Postpartum samples were collected before pre-dipping disinfectant solutions were applied to the teat and before milk samples were collected.

5.3.4 Teat apex culture and species identification

The methods for culture and species-level identification were adapted from protocols utilized in the Laboratory for Udder Health at the University of Minnesota to process towel samples (Rowe et al., 2019). Before sample processing and to reduce the possibility of batch effects, sample IDs were randomly sorted using Excel (Microsoft Corporation, Redmond, WA, USA). In accordance with this order, isolates were selected for each sampling processing date in batches of 10 isolates. Gauze samples were thawed at 4 °C overnight and then transferred into 50 mL round-bottom falcon tubes (Corning Inc.). Afterwards, 5 mL of Phosphate-Buffered Saline (**PBS**; Gibco, Termofisher Scientific) was added to reduce viscosity. Samples were homogenized carefully by turning tubes upside down twice and vortexing for 10 seconds at low speed. Samples were allowed to stand for 10 minutes, and the homogenization process was repeated. Following homogenization, one 1 in 10 serial dilution was done using PBS. A 100 uL aliquot from each sample (i.e., undiluted and 10^{-1}) was inoculated onto Mannitol salt agar (Oxoid). Plates were then incubated in aerobic conditions for 24 hours at 37 °C. Following incubation, bacterial groups were visually assessed and counted from the dilution plate with the optimal number of colonies (25 to 250 per plate). Isolates were then picked from the plate using a sterile calibrated loop (Thermofisher Scientific), streaked onto blood agar (Hardy Diagnostics) and incubated for 24 hours at 37 °C.

5.3.5. Species identification of isolates

After reaching pure growth, the identity of representative colonies was further
investigated using a Matrix-Assisted Laser Desorption/Ionization-Time of Flight (**MALDI-
TOF**) mass spectrometer (**MS**) (Microflex; BrukerDaltonics Inc.) (Jahan et al., 2021). Mass
spectra profiles produced from each isolate were matched against the Biotyper reference library.
Confidence scores were used to assign genus- and species-level classifications (≥1.8 and ≥ 2.0
for genus- and species- classification, respectively) (Jahan et al., 2021). Non-*aureus*
Staphylococci and closely related *Mammaliicoccal* species isolates were then stored for further
investigation in -80 °C using commercial cryovials (Hardy Diagnostics). Isolates identified as
NASM using MALDI-TOF MS were further investigated using whole genome sequencing.
Isolates were first grown on brain heart infusion for 24 hours, centrifuged at 10,000 rpm for 5
minutes, and the pellet was collected in 2 mL sterile polypropylene microcentrifuge tubes
(Thermo Fisher Scientific). Following this, DNA extraction was performed utilizing commercial
kits and following manufacturer's protocol (Qiagen's DNeasy 96 Blood & Tissue Kit, Qiagen).
Amount and quality of extracted DNA was evaluated using PicoGreen fluorometer and
NanoDrop spectrophotometer (Thermo Fisher Scientific). Library preparation was performed
using the DNA Prep Sample Preparation Kit (Illumina Inc.), and libraries were pooled for 2x300
paired-end sequencing on the Illumina MISEQ platform, with a targeted depth of 50x coverage
across the *Staphylococcus* spp. genome for each library. Quality control of raw sequencing data
was performed using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016). Trimmomatic
was utilized to trim low quality bases and adapters from raw reads (Bolger et al., 2014).
Genomes were assembled utilizing SPAdes version 3.15.5 (Bankevich et al., 2012), and

assembly quality was evaluated using Quast version 5.2.0 (Gurevich et al., 2013). Species identification of isolates was performed utilizing GTDB-Tk version 1.7.0 (Chaumeil et al., 2020) and GTDB release 202 (Parks et al., 2022) as implemented in KBase (Arkin et al., 2018).

5.3.6. Milk sample collection

After calving, quarter milk samples were collected weekly during the first 5 postpartum weeks following guidelines from National Mastitis Council (NMC, 2017). After teat apex samples were collected, a pre-dipping disinfectant solution was applied. Upon removal of the pre-dipping disinfectant, 3 to 4 streams of milk were discarded from each quarter, and the teat apex of each quarter was scrubbed with a gauze soaked in 70% ethanol. Milk samples were collected in a clockwise direction, beginning with the right rear quarter and ending with the right front quarter. Approximately 10 mL of milk from each quarter were collected into separate 60 mL plastic vials (Capitol Vials, Termo Fisher). Samples were placed on ice immediately after collection and stored in a freezer at -20 °C until further processing.

5.3.7. Milk pooling

Quarter milk samples were combined into composite samples using the following protocol (Peña-Mosca et al., 2023b). First, milk samples were thawed at 4 °C overnight. Following, quarter milk samples were homogenized by inverting them upside down twice. Then, 2 mL was extracted from each vial and dispensed into a single plastic vial inside a laminar hood. Lab-pooled composite samples were then submitted to the Laboratory for Udder Health at the University of Minnesota (St. Paul, MN, USA) for milk culture.

5.3.9. Milk culture

Using a cotton swab, approximately 100 μL of milk were plated onto Columbia CNA agar with 5% sheep blood and MacConkey agar. Agar plates were incubated in aerobic conditions at 37 °C for a total of 42 to 48 h. Plates were examined by a trained technician to evaluate the presence of growth of distinct isolates at 18 to 24 hours and 42 to 48 hours. Samples were defined as contaminated if more than 3 different isolates were identified and omitted from further analysis (Dean et al., 2022). Taxonomic assignments of cultured isolates were made using a MALDI-TOF MS as previously described for teat-apex samples. An IMI was defined as a composite sample containing one or more than one colony forming units (10 colony forming units (**CFU**)/mL) of any cultured isolates. Microorganisms were grouped according to their morphological and epidemiological characteristics in different bacterial groups: SAU, NASM (members from *Staphylococcus* genus that were not SAU), SSLO which include members from *Streptococcus* genus, as well as *Streptococcus*-like organisms (e.g., *Enterococcus* spp., *Aerococcus* spp., *Lactococcus* spp. and *Micrococcus* spp.), gram negative microorganisms (e.g., *Escherichia* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Enterobacter* spp., *Pantoea* spp.) and others (microorganisms that did not belong to any of the previous groups).

5.3.10. Determination of in vitro antimicrobial activity of NASM isolates using the agar dilution method

Methods utilized in this manuscript were based on previously described methods (Toledo-Silva et al., 2022). Each week, on day 1, isolates were randomly sorted using Excel (Microsoft Corporation). Following this, isolates that were previously identified using MALDI-TOF MS as NASM were grown for 24 hours in blood agar (Hardy Diagnostics). If evidence of

mixed growth (more than 1 colony type) was present, samples were replated. On day 2, one colony was picked with a 10 uL disposable loop and was grown on brain heart infusion (**BHI**; Research Products International Corporation) media for 18 to 20 hours. The next day, 1 in 10 serial dilutions solutions were created using sterile PBS in a 96 well plate (Thermo Fisher Scientific). Bacterial count (CFU/mL) was estimated by plating 100 uL of the serial dilutions on blood agar plates that were then aerobically incubated for 18 to 20 h at 37 °C. One hundred microliters of bacterial suspensions containing NASM isolates at different dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) were added to 10 mL of 2% Tryptic soy agar (**TSA**; Oxoid) inside Falcon round-bottom polypropylene tubes (Corning Inc.). The temperature was examined using a laboratory digital thermometer (Thermco) before the utilization of 2% TSA. The media were not used until the temperature reached 50 °C (Toledo-Silva et al., 2022). Tubes were then homogenized and poured into pre-heated (37 °C) TSA plates. Following aerobic incubation for 18 to 20 h, 10 uL of the bacterial suspensions containing approximately 10,000 CFU of SAU (American type culture collection (**ATCC**) 25923) and SUB (ATCC 19436) were inoculated onto the top of plates containing different concentrations of NASM isolates and incubated for 18 to 20 h. For each batch of samples, negative controls were included by incubating a 2% TSA agar plate with the inclusion of 100 uL of BHI only (instead of NASM isolates). Additionally, a 10 μL droplet of PBS was added on top of the media on each plate to serve as an additional negative control, ensuring the absence of PBS contamination. Growth of SAU and SUB (i.e., yes vs no) was evaluated by visual examination of each of the plates and comparison with negative control plates within each batch. Minimum inhibitory concentration (**MIC**) was defined as the

minimum concentration of NASM (CFU/mL) on the 2% TSA top layer able to inhibit the growth of SAU or SUB.

5.3.11. Statistical analysis

All analyses were performed in R 4.3.2 (https://www.r-project.org/). Code and output can be found online: https://fepenamosca.github.io/NASM_in_vitro_inhibitory_activity.io/ Before modeling, descriptive characteristics for numerical variables and categorical outcomes were evaluated using the "table 1" package in R (Rich et al., 2023). Data distribution was evaluated by the use of density plots as implemented in the "car" package in R (Fox et al., 2022). The relationship between IMI status (i.e., cases versus controls) and log_{10} -transformed bacterial counts from teat apex samples was investigated using linear regression models. The relationship between IMI status and growth (yes or no) of NASM from the teat apex samples was investigated using a generalized linear model (logistic regression) with a "binomial" family and "logit" link. For analysis, NASM taxonomy was determined based on the assigned taxonomy using MALDI-TOF MS and WGS. It was represented as a six-level multi-level variable (*Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus sciuri*, *Staphylococcus succinus*, *Staphylococcus xylosus/pseudoxylosus*, other rare NASM [*Staphylococcus cohnii*, *Staphylococcus devrieseii*, *Staphylococcus equorum*]). To assess the association between NASM taxonomy and NASM *in vitro* growth inhibition on SUB and SAU*,* Cox proportional hazards regression was utilized. Observations were right censored when inhibition was still not present at maximum NASM concentration analyzed (i.e., 10^{-7} dilution). Proportional hazards assumption was tested by evaluating the Schoenfeld residuals. Since multiple isolates were harbored from each cow, the non-independence of observations within

each enrolled animal was accounted for using the robust sandwich estimator. The NASM MIC ($\log_{10}$, CFU/mL) on SAU and SUB was further explored and compared across cases and controls and different NASM species using mixed linear regression, incorporating cow ID as a random effect to address the correlation between observations within the same cow. Following this, isolates were ranked according to their MIC (e.g., 1 to 79) and classified as being in the top 10 isolates with lowest MIC or not ("top 10"). The relationship between IMI status and presence of a "top 10" NASM on the teat apex was investigated using a generalized linear model (logistic regression) with "binomial" family and "logit" link. When performing this analysis by NASM species, we used the Fisher exact test due to the absence of "top 10" isolates for some NASM species. The intraclass correlation coefficient was estimated using the "performance" package (Lüdecke et al., 2021). Estimated marginal means were estimated using the "emmeans" package in R (Lenth et al., 2022). Normality assumption was investigated by evaluation of model residuals and the use of quantile-quantile plots. Multiple comparisons were accounted for with Tukey adjustment as implemented in the "emmeans" package (Lenth et al., 2022). Farm-ID was forced into all models that investigated the relationship between IMI status and NASM inhibitory activity against SAU and SUB. Because farm-ID was strongly associated with the prevalence of different NASM species, this variable was not included in the models investigating the association between NASM taxonomy and inhibitory activity.

For all models, processing batch (i.e., the date at which gauze samples were processed or antimicrobial activity of NASM was evaluated) and parity of the cow (i.e., first vs second lactation) were offered as potential confounders. Presence of confounding was evaluated by examining the change in the estimates after adjustment by each confounder ([Crude estimate –

Adjusted estimate] / Adjusted estimate). Confounders that changed the estimates by 10% or more were kept in the models.

5.4. RESULTS

5.4.1. Descriptive characteristics

Farms enrolled in this study were located in Minnesota (farm A) and Colorado (farm B) with herd sizes of 100 and 3,000 milking cows, respectively. Both farms utilized a free-stall housing system and cows consumed at least 30% of DMI from pasture, when possible, following organic regulations. All cows in farm A (14/14) were starting their first lactation, while for farm B 46.2% (43/93) of the cows were starting their first lactation and 53.8% (50/93) were transitioning to their second lactation. A total of 68 animals (34 cases and 34 controls) were included in this case control study (**Table 5.1**). Fifteen of these cases had a SAU-IMI, 15 had a SSLO-IMI and 4 had an IMI caused by both microorganisms. In the first postpartum sample, 64.7% (22/34) of the cases had the presence of a SAU or SSLO IMI. Teat apex samples processed from cases and controls showed similar days relative to calving (mean [SD]; Controls: -2.8 [15.2] vs Cases: -1.2 [16.5]). A flowchart describing the *in vitro* study as well as the number of observations lost at each step is presented in **Figure 5.1**.

5.4.2. *In vitro inhibitory activity of non-aureus Staphylococci and closely related Mammaliicoccal species isolated from case and control cows against Staphylococcus aureus and Streptococcus uberis*

A total of 275 isolates were submitted for MALDI-TOF MS as shown in **Table 5.2**. Surprisingly, *Bacillus* spp. were the most frequently identified genus in controls (61.9%

[83/134]) and cases (61.7% [87/141]). Non-*aureus Staphylococci* and closely related

Mammaliicoccal species were identified in 32.8% (44/134) and 27.7% (39/141) of the isolates

obtained from the teat apex samples from controls and cases, respectively. The most frequent

species identified within this group included *Staphylococcus chromogenes* (8.0% [22/275]) and

Staphylococcus haemolyticus (7.6% [21/275]). The overall inhibitory activity of NASM on SAU

was not different between isolates obtained from cases and controls (HR (95%CI): 0.92 (0.56,

1.49), *P*= 0.72; **Figure 5.2**). The adjusted MIC (log_{10}, CFU/mL) for SAU was 3.26 and 3.37 for

isolates recovered from controls and cases, respectively (Estimate [95%CI]: 0.11 [-0.21, 0.44];

P=0.49**)**. Similarly, we did not find evidence of a difference in the inhibitory activity of NASM

on SUB between isolates obtained from cases and controls (HR (95%CI): 1.23 (0.74, 2.08),

P=0.45; **Figure 5.2**). The adjusted MIC (log_{10}, CFU/ml) of NASM against SUB was 3.17 in

isolates from controls and 3.08 in isolates from cases (Estimate [95%CI]: -0.10 [-0.45, 0.25];

P=0.58).

In the course of the study, we evaluated the inhibitory activity of 79 NASM isolates. We

were not able to ascertain the MIC against SAU and SUB for 5 and 14 NASM isolates,

respectively, because these isolates did not show any inhibitory activity even at the highest

concentration analyzed (**Figure 5.3**). Among these isolates, the average highest concentration

with an undetermined MIC was 3.98 (0.45) CFU/mL for SAU and 4.30 (0.52) CFU/mL for SUB.

To explore isolates with high inhibitory activity, we arranged the isolates based on their MIC

values against SAU and SUB (**Figure 5.3A and 5.3B**). The 10 isolates with lowest MICs

displayed a mean (SD) MIC (log_{10}, CFU/ml) of 2.41 (0.26) and 2.16 (0.32) for SAU and SUB,

respectively. These isolates showed MICs that ranged between 1.92 to 2.66 log_{10} CFU/mL

against SAU growth and 1.65 to 2.56 $\log_{10}$ CFU/mL against SUB growth. Top 10 isolates with lowest MIC against SAU were more prevalent in controls (19.4% (7/36)) than in cases (5.8% (2/35)) (OR (95%CI): 0.25 (0.04, 1.13), P=0.07). However, no evidence of an association was found between IMI status and the likelihood of harboring a 10 top isolate for SUB (controls: 11.1% (4/35) vs cases: 14.7% (5/36); (OR (95%CI): 1.33 (0.32, 5.83), P=0.69).

5.4.4. Relationship between case control status and bacterial counts on teat apex

The association between IMI status and bacterial count in the teat apex samples is presented in **Table 5.3**. No evidence of a difference in NASM counts was found between cases and controls (Estimate [95%CI]: -0.09 [-0.51, 0.36], P=0.72). The likelihood of harboring NASM on the teat apex was not different between controls (66.7% [22/36]) and cases (62.9% [24/35]) (OR (95%CI): 0.85 (0.32, 2.25), P=0.74) (**Table 5.4**). The distribution of NASM species differed by farm. *Staphylococcus chromogenes, Staphylococcus haemolyticus* and *Staphylococcus succinus* showed a high prevalence in farm A (28.1% [16/57], 28.1% [16/57] and 14.1% [8/57], respectively), but were rarely found on farm B (7.1% [1/14], 7.1% [1/14] and 0% [0/14], respectively). On the other hand, *Staphylococcus xylosus* or *Staphylococcus pseudoxylosus* were found in half of the samples from farm B (50.0% [7/14]) but showed a low prevalence in farm A (1.8% [1/57]).

5.4.5. Relationship between non-aureus Staphylococci and closely related Mammaliicoccal species taxonomy and inhibitory activity against Staphylococcus aureus and Streptococcus uberis

The inhibitory activity of NASM on SAU and SUB differed across different NASM species ($P < 0.001$; **Figure 5.3, Figure 5.4**). *Staphylococcus haemolyticus* and other rare NASM species (i.e., *Staphylococcus cohni*, *Staphylococcus devrieseii*, *Staphylococcus equorum*) showed lower inhibitory activity against SAU compared to *Staphylococcus chromogenes* (HR [95%CI]: 0.28 [0.09, 0.90] and HR [95%CI]: 0.15 [0.03, 0.71], respectively), *Staphylococcus sciuri* (HR [95%CI]: 0.21 [0.08, 0.58] and HR [95%CI]: 0.11 [0.02, 0.50], respectively), *Staphylococcus succinus* (HR [95%CI]: 0.15 [0.05, 0.42] and HR [95%CI]: 0.08 [0.02, 0.36], respectively) and *Staphylococcus xylosus*/*pseudoxylosus* (HR [95%CI]: 0.14 [0.05, 0.38] and HR [95%CI]: 0.07 [0.02, 0.28], respectively). The adjusted MIC ($\log_{10}$ CFU/ml) of NASM on SAU for different NASM species ranged between 2.92 and 3.99 (**Table 5.5**). Similarly, when inhibition of SUB was the outcome of interest, *Staphylococcus haemolyticus* displayed a reduced ability to inhibit SUB compared to *Staphylococcus chromogenes* (HR [95%CI]: 0.11 [0.02, 0.49]), *Staphylococcus sciuri* (HR (95%CI): 0.17 (0.06, 0.48)), *Staphylococcus succinus* (HR [95%CI]: 0.13 [0.03, 0.50]) and *Staphylococcus xylosus*/*pseudoxylosus* (HR [95%CI]: 0.11 [0.03, 0.43]). However, the inhibitory activity of *Staphylococcus haemolyticus* against SUB was not different from other rare NASM species (e.g., *Staphylococcus cohnii*, *Staphylococcus devriesei*, *Staphylococcus equorum*) (HR [95%CI]: 0.62 [0.06, 6.31]). The adjusted MIC ($\log_{10}$ CFU/ml) of NASM against SUB in different NASM species ranged between 2.92 and 3.63 (**Table 5.5**). The distribution of isolates ranked by their MIC on SAU and SUB growth is shown in **Figure 5.3C**

and 5.3D. The proportion of isolates classified as "top 10" with respect to SAU and SUB was different between the NASM species (*P*=0.04 and 0.06, respectively). Only 4.8% (1/21) and 0% (0/5) of the isolates identified as *Staphylococcus haemolyticus* and other rare NASM species, respectively, were classified as being "top 10" against both SAU and SUB. By comparison, 45.5% (5/11) of *Staphylococcus succinus* isolates were classified as "top 10" against both SAU and SUB (**Table 5.6**).

5.6. DISCUSSION

This study aimed to explore the association of naturally occuring NASM isolates and their ability to inhibit the growth of SAU and SUB, two major mastitis pathogens that are difficult to control and eradicate on dairy farms (Barkema et al., 2006; Peña-Mosca et al., 2023b; Woudstra et al., 2023). In our study, we observed an association between the presence of isolates with high *in vitro* antimicrobial activity against SAU and absence of IMI by SAU or SSLO. In addition, the inhibitory activity of NASM showed substantial variation across different NASM species.

5.6.1. Relationship between in vitro antimicrobial activity of NASM isolates and presence of intramammary infections

Our primary objective was to investigate the *in vitro* antimicrobial activity of NASM isolates and their association with the presence of IMI. We discovered a wide variation in the inhibitory activity across NASM isolates. A considerable number of the NASM isolates did not show inhibitory activity at the maximum concentration studied suggesting the absence of important inhibitory activity for those microorganisms. Moreover, we did not find evidence of a

difference in the average inhibitory activity of NASM on SAU or SUB across cases and controls. One possible explanation for these findings is that average antimicrobial activity may not be an important predictor of the risk of IMI, and only high antimicrobial activity against a specific pathogen may be effective to prevent the risk of IMI (Nakatsuji et al., 2017). In this study, we discovered that control cows had the majority of NASM isolates that were within the "top 10" with the lowest MIC against SAU. In addition, cows in the control group had a higher chance of containing these highly inhibitory isolates on their teat apices than cows in the case group. Although our results need to be interpreted with caution, given the small sample size and imprecision of estimates, these results suggest the presence of a protective effect due to the presence of NASM with high in-vitro antimicrobial activity against SAU on the presence of IMI by SAU or SSLO. These findings are in line with studies performed in humans in which isolates with high phenotypic antimicrobial activity showed a high prevalence on the skin on controls, but were rarely identified on people with SAU skin infections (Nakatsuji et al., 2017). As whole, our results suggest that commensal NASM may provide defense against mammary gland pathogen invasion, albeit these research discoveries require more study.

5.6.2. Relationship between teat apex colonization by non-aureus Staphylococcus and closely related Mammaliicoccal species and presence of intramammary infections

The prevalence of NASM on teat apex (64.8%) was similar to that reported by other studies in conventional dairy farms in which 70.1 % (Mahmmod et al., 2018) and 71.8% (De Visscher et al., 2016b) of the teat-apex samples showed presence of NASM. Consistently, we found a high prevalence of *Staphylococcus chromogenes* and differences in the prevalence of the different NASM species across the enrolled farms, which concurs with these previous reports

(De Visscher et al., 2016b; Mahmmod et al., 2018). These differences have been previously attributed to differences in management across the enrolled farms (De Buck et al., 2021) and are not surprising, given the differences in the prevalence and distribution of different microorganisms that cause IMI in organic dairy farms (Peña-Mosca et al., 2023b).

Contrary to our hypothesis, the counts or the presence of NASM on teat apex were not associated with the presence of SAU or SSLO IMI. These results are opposite to those reported by prior studies, in which prepartum colonization of teat apices by NASM was related to a lower risk of postpartum IMI (Piepers et al., 2011) and subclinical mastitis (De Vliegher et al., 2003). These findings suggest that, in our study population, the mere presence of NASM on the teat apex was not a significant factor driving the risk of IMI in early lactation cows. Other factors, which could potentially account for NASM's ability to prevent major pathogens IMI, as has been proposed in previous research (Reyher et al., 2012), including the presence of *in vitro* antimicrobial activity (Braem et al., 2014; Carson et al., 2017; Toledo-Silva et al., 2022).

5.6.3. Relationship between non-aureus Staphylococcus and closely related Mammaliicoccal species taxonomy and presence of in vitro antimicrobial activity

In our study, we were able to identify 9 different NASM species isolated from teat apices of organic dairy cows. Consistently with previous studies we found important differences across different NASM species (Braem et al., 2014; Carson et al., 2017). In these studies, the assessment of NASM's antimicrobial activity has relied on the cross-streak method. This methodology revealed that between 9.1% and 13% of the isolates showed signs of antimicrobial activity (Braem et al., 2014; Carson et al., 2017). In our study we opted to use agar dilution methods (Wiegand et al., 2008). A methodology that shows results that not only concurs with the

cross-streak technique, but also provides a quantitative measurement of the antimicrobial activity of bacterial isolates (Toledo-Silva et al., 2022). To the best of our knowledge, the quantitative method has only been investigated in 3 different NASM species isolates from the udder of dairy cows, which included *Staphylococcus chromogenes*, *Staphylococcus epidermidis* and *Staphylococcus simulans*. The MIC of these species against *Staphylococcus aureus* ranged between 5.10 and 8.24 CFU/mL (log_{10}) (Toledo-Silva et al., 2022). In our study, we observed that the inhibitory activity of NASM on SAU or SUB was different across different species. Our results suggested that *Staphylococcus haemolyticus* possesses little to no inhibitory activity on SAU or SUB. This finding is inconsistent with earlier research that showed that SAU was not inhibited by *Staphylococcus haemolyticus* (Carson et al., 2017). In contrast, *Staphylococcus succinus* exhibited the lowest MIC values, with almost fifty percent of these isolates falling within the "top 10" isolates possessing the lowest MIC. This differs from other groups, where this proportion did not exceed 20%. In addition, this contradicts prior research showing no inhibitory activity of *Staphylococcus succinus* against SAU (Carson et al., 2017). These results may be explained by the observation that antibacterial activity in earlier research in humans appeared to be strain-specific (Nakatsuji et al., 2017). Hence, it is plausible that antimicrobial activity of NASM could be attributed more to specific strains rather than distinct species. Investigating the antimicrobial activity across different strains within each of the NASM species was beyond the scope of this project and should be further investigated in the future.

5.6.4. Study validity and limitations

This study investigated the quantitative antimicrobial activity of NASM isolates against SAU and SUB. While this method represents a novel approach that has only recently been

described for its use on mastitis pathogens (Toledo-Silva et al., 2022), it also has certain limitations. For instance, the novelty of these methods in mastitis research, coupled with the absence of predetermined MIC cut-off points, complicates the interpretation of results. This makes it challenging to discern whether the absence or presence of inhibition is due to antimicrobial activity or merely a competition for nutrients that creates unfavorable conditions for the growth of target microorganisms in the media (De Vliegher et al., 2004c; Toledo-Silva et al., 2022).

Additional steps to control for potential technical and biological confounders, including the randomization of samples for processing, matching by important confounders, and the assessment of the magnitude of confounding by the inclusion of these variables into the models were used in this study. Nonetheless, it is vital to understand that some of our results could be still biased by other unmeasured confounders. For instance, cow-level factors that increased the likelihood of presence of IMI such as abnormally high or low body condition score, milk leakage (Fernandes et al., 2022), dysfunctional immune response (Sordillo, 2018), or dirty udders (Schreiner and Ruegg, 2003; Compton et al., 2007b) could have influenced our results. Moreover, the small sample size and therefore imprecision of estimates could hinder our ability to make inference from the results in this exploratory study.

Lastly, one major limitation of our study was the fact that almost two thirds of SAU and SSLO IMI were evident in the first postpartum milk sample. This suggests that IMI may have been acquired before calving. This finding is not surprising given the challenges to control mastitis without antibiotics (NMC, 2019) and the increased prevalence of SAU on organic dairy farms (Pol and Ruegg, 2007; Cicconi-Hogan et al., 2013). All the above limits our ability to

determine if colonization of NASM with antimicrobial activity preceded the acquisition of SAU or SUB IMI. Therefore, it is conceivable that other factors not explored in this study, such as the presence of a close-up pen with poor bedding management, high stocking density, high fly presence, and presence of IMI carried across lactations for multiparous cows, that may be influencing the results and explaining why some of these animals calved with IMI (Green et al., 2008; De Vliegher et al., 2012; Fernandes et al., 2022).

5.6.5. *External validity*

This study was conducted in two organic dairy farms located in Minnesota and Colorado. Considering that the prevalence of different NASM species differs substantially across different dairy farms in this and previous studies (De Visscher et al., 2016b; Mahmmod et al., 2018), we consider that results could only be generalized to dairy farms managed under similar conditions. In addition, because antimicrobial activity differed across different NASM species and even within the species, results should be confirmed including a larger number of isolates representative of different species that can be found on the mammary gland of dairy cows. Finally, to address the wide heterogeneity of NASM antimicrobial activity, strain-level identification is required, in order to identify clades of bacteria and antimicrobial peptides connected to the presence of *in vitro* antimicrobial activity.

5.7. CONCLUSIONS

The presence of NASM isolates classified as "top 10" with lowest MIC against SAU was highest in cows without SAU or SUB IMI (controls) compared to cows that had an IMI by these microorganisms (cases), however, no evidence of an association was found between presence of

isolates with "top 10" lowest MIC against SUB and IMI status. In this study, we found no evidence of an association between NASM counts on teat apex and presence of IMI by SAU or SSLO. The inhibitory activity of NASM was different across different NASM species. This study provides initial information about the inhibitory capabilities of naturally occurring NASM isolated against SAU and SUB and contributes to our understanding of the relationship between NASM and udder health in dairy cows.

5.8 TABLES AND FIGURES

5.8.1. Tables

Table 5.1. Descriptive characteristics of enrolled cases (n=34) and controls (n=34).

Item	Controls	Cases
Farm		
A	20.6% (7/34)	20.6% (7/34)
B	79.4% (27/34)	79.4% (27/34)
Parity		
First lactation	47.1% (16/34)	47.1% (16/34)
Second lactation	52.9% (18/34)	52.9% (18/34)
Case type		
Staph aureus only	-	44.1% (15/34)
SSLO only	-	44.1% (15/34)
SSLO and *Staph aureus*	-	11.8% (4/34)
Cows positive at calving	-	64.7% (22/34)
Days in milk new IMI	-	13.2 (12.0)
Days relative to calving teat apex sample †	-2.8 (15.2)	-1.2 (16.5)

Cows positive at calving: Cows with an IMI by *Staphylococcus aureus* or *Streptococcus* spp. and *Streptococcus*-like organisms (SSLO) on the first postpartum sample. † Teat apex sample: gauze sample extracted on the week prior to the first time a cow tested positive for an IMI.

Table 5.2. Bacterial counts on teat-apex ($\log_{10}$ colony forming units/square inch of gauze) of controls (referent) and cases (n=71 teat apex samples).

Outcome	Estimated marginal means (SE)		Estimate (95%CI)	*P-value*
	Controls	Cases		
Total bacterial count	1.81 (0.12)	1.91 (0.12)	0.10 (-0.19, 0.40)	0.50
NASM	1.12 (0.18)	1.02 (0.18)	-0.09 (-0.54, 0.35)	0.67
SCH	0.26 (0.12)	0.20 (0.12)	-0.05 (-0.34, 0.23)	0.71
SHAEM	0.21 (0.13)	0.21 (0.13)	-0.005 (-0.33, 0.32)	0.98
SSC	0.19 (0.10)	0.21 (0.10)	0.02 (-0.23, 0.27)	0.86
SSUC [a]	0.17 (0.09)	0.17 (0.09)	-0.004 (-0.24, 0.24)	1.00
SXYL	0.46 (0.09)	0.53 (0.10)	0.06 (-0.17, 0.30)	0.60
Other	0.20 (0.07)	0.12 (0.07)	-0.08 (-0.26, 0.09)	0.35

[a] *Staphylococcus succinus* (SSUC): Model did not account for farm, because it was not present in farm B. NASM: non-*aureus Staphylococci* and closely related *Mammaliicoccal* species. SCH: *Staphylococcus chromogenes*. SHAEM: *Staphylococcus haemolyticus*. SSC: *Staphylococcus sciuri*. SXYL: *Staphylococccus xylosus/pseudoxylosus*. Other: *Staphylococcus cohnii, Staphylococcus devreseii* and *Staphylococcus equorum.*

Table 5.3. Presence of non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) isolates on teat-apex of controls (referent) and cases (n=71 teat apex samples).

Outcome	Presence on teat apex		OR (95%CI)	*P-value*
	Controls	Cases		
NASM	66.7% (24/36)	62.9% (22/35)	0.85 (0.32, 2.25)	0.74
SCH	25.0% (9/36)	22.9% (8/35)	0.89 (0.29, 2.73)	0.84
SHAEM	25.0% (9/36)	22.9% (8/35)	0.89 (0.29, 2.73)	0.84
SSC	8.3% (3/36)	5.7% (2/35)	0.66 (0.08, 4.28)	0.66
SSUC [a]	11.1% (4/36)	11.4% (4/35)	1.03 (0.23, 4.72)	0.97
SXYL	11.1% (4/36)	17.1% (6/35)	2.17 (0.39, 14.47)	0.38
Other	8.3% (3/36)	2.9% (1/35)	0.31 (0.01, 2.63)	0.29

[a] *Staphylococcus succinus* (SSUC): Model did not account for farm, because it was not present in farm B. SCH: *Staphylococcus chromogenes*. SHAEM: *Staphylococcus haemolyticus*. SSC: *Staphylococcus sciuri*. SXYL: *Staphylococccus xylosus/pseudoxylosus.* Other: *Staphylococcus cohnii, Staphylococcus devreseii* and *Staphylococcus equorum.*

Table 5.4. Taxonomic identification of isolates harbored from teat apex samples and submitted for taxonomic identification using Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass spectrometer (n=275 isolates) and confirmation for non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) using whole genome sequencing (n=83 isolates).

Taxonomy classification	Controls	Cases
Staph aureus	0% (0/134)	1.4% (2/141)
NASM	32.8% (44/134)	27.7% (39/141)
Staph chromogenes	9.0% (12/134)	7.1% (10/141)
Staph cohnii	0% (0/134)	0.7% (1/141)
Staph devreseii	1.5% (2/134)	0% (0/141)
Staph equorum	0.7% (1/134)	0% (0/141)
Staph haemolyticus	9.0% (12/134)	6.4% (9/141)
Staph pseudoxylosus	1.5% (2/134)	2.1% (3/141)
Staph sciuri	2.1% (3/134)	2.1% (3/141)
Staph succinus	4.5% (6/134)	4.3% (6/141)
Staph xylosus	4.5% (6/134)	5.0% (7/141)
Aerococcus viridans	0% (0/134)	1.4% (2/141)
Unspeciated *Aerococcus*	0.8% (1/134)	2.8% (4/141)
Bacillus spp.	61.9% (83/134)	61.7% (87/141)
Gram-positive rod	4.5% (6/134)	5.0% (7/141)

Table 5.5. Relationship between species identification and minimum inhibitory concentration (MIC) of non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) against *Staphylococcus aureus* and *Streptococcus uberis* (n=79 isolates).

NASM taxonomy	*Staphylococcus aureus*		*Streptococcus uberis*	
	MIC (SE)	*P-value*	MIC (SE)	*P-value*
Staph chromogenes	3.32 (0.13) [a]	0.001	3.06 (0.16)	0.23
Staph haemolyticus	3.99 (0.14) [b]		3.63 (0.26)	
Staph sciuri	3.32 (0.24) [a]		3.49 (0.30)	
Staph succinus	2.92 (0.17) [a]		2.92 (0.22)	
Staph xylosus/pseudoxylosus	3.05 (0.14) [a]		3.05 (0.17)	
Other	4.39 (0.32) [b]		3.29 (0.49)	

Other NASM: *Staph cohnii, Staph devreseii, Staph equorum.* Different super indexes within the same column indicate statistical differences. Intraclass correlation coefficient cow-ID *Staph aureus*: Not available, *Strep uberis*: Not available. Other: *Staphylococcus cohnii, Staphylococcus devreseii* and *Staphylococcus equorum.* Only isolates in which MIC was determined were included for analysis; this included a total of 75 isolates for *Staphylococccus aureus*: *Staphylococcus chromogenes:* 100% (19/19), *Staphylococcus haemolyticus*: 81.0% (17/21), *Staphylococcus sciuri*: 100% (6/6), *Staphylococcus succinus*: 100% (11/11), *Staphylococcus xylosus/pseudoxylosus*: 100% (18/18), other NASM: 75.0% (3/4). For *Streptococcus uberis* MIC was determined in 63 isolates: *Staphylococcus chromogenes:* 100% (19/19), *Staph haemolyticus*: 33.0% (7/21), *Staphylococcus sciuri*: 100% (6/6), *Staphylococcus succinus*: 100.0% (11/11), *Staphylcooccus xylosus/pseudoxylosus*: 100% (18/18), other NASM: 50.0% (2/4).

Table 5.6. Relationship between non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) identified species and isolates classification as the "top 10" with the lowest minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Streptococcus uberis* (n=79 isolates).

Outcome	NASM taxonomy	Presence of isolates "top 10" lowest MIC	*P-value*
Inhibition *Staph aureus*	*Staph chromogenes*	10.5% (2/19)	0.04
	Staph haemolyticus	4.8% (1/21)	
	Staph sciuri	16.9% (1/6)	
	Staph succinus	45.5% (5/11)	
	Staph xylosus/pseudoxylosus	5.6% (1/18)	
	Other	0.0% (0/4)	
Inhibition *Strep uberis*	*Staph chromogenes*	10.5% (2/19)	0.06
	Staph haemolyticus	4.8% (1/21)	
	Staph sciuri	0% (0/6)	
	Staph succinus	45.5% (5/11)	
	Staph xylosus/pseudoxylosus	11.1% (2/18)	
	Other	0.0% (0/4)	

Other: *Staphylococcus cohnii, Staphylococcus devreseii* and *Staphylococcus equorum.*
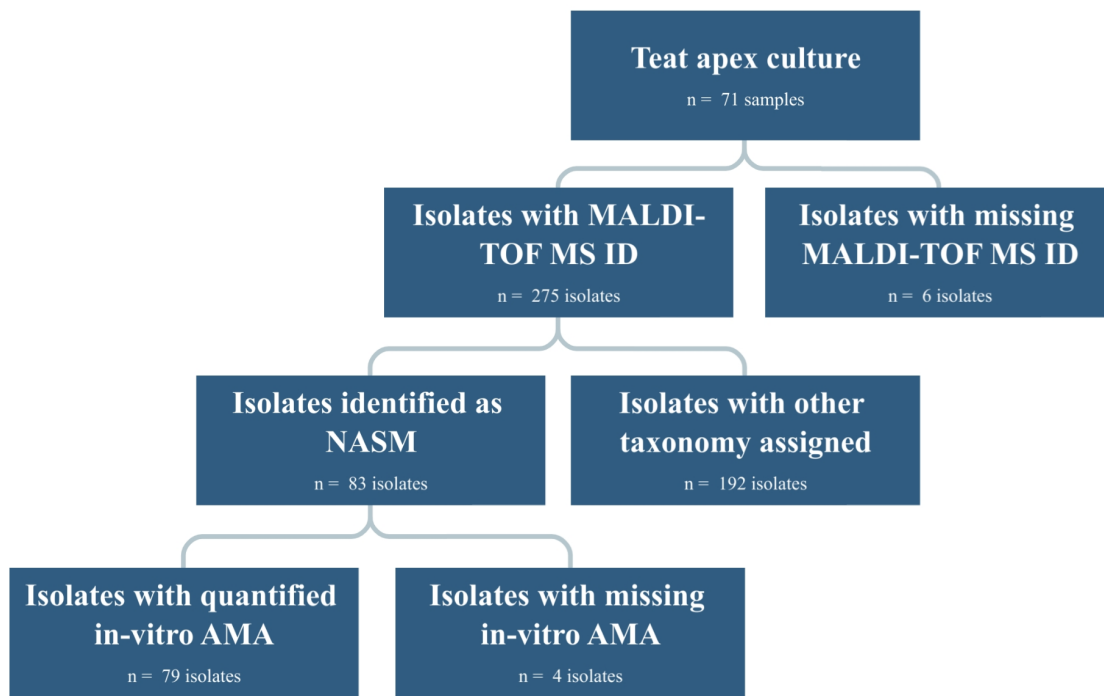
Figure 5.1. Outline of the study with number of observations lost at each step. NASM: non-*aureus Staphylococci* and closely related *Mammaliicoccal* species. AMA: antimicrobial activity.
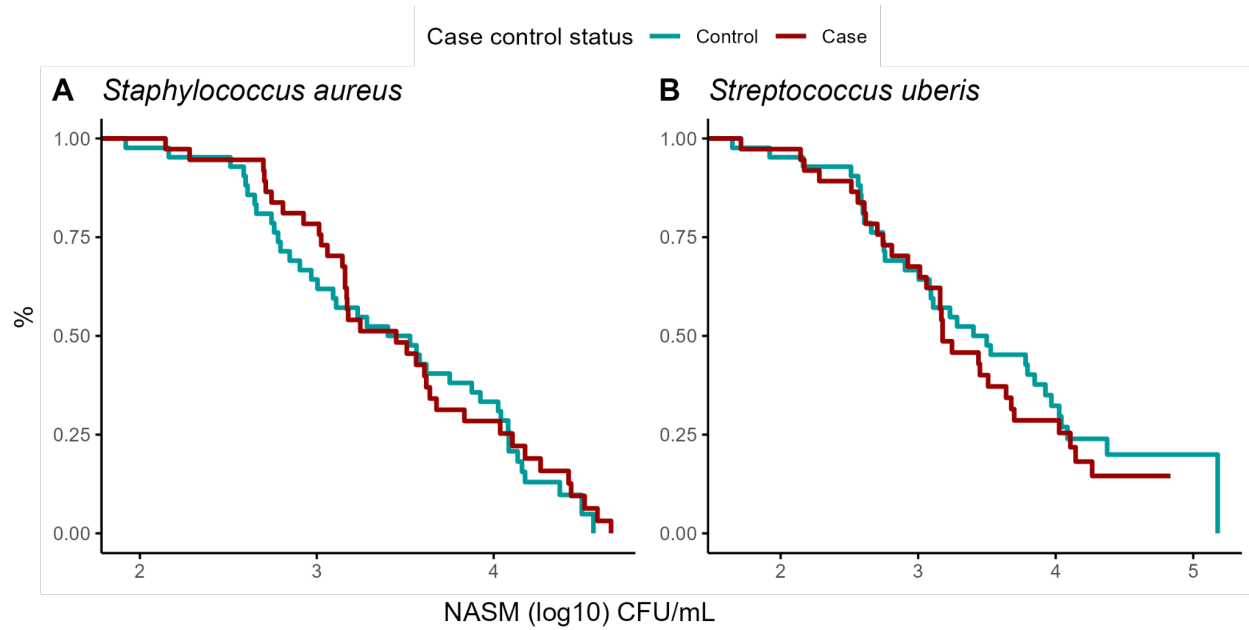
Figure 5.2. Kaplan meier plots showing presence of growth of *Staphylococcus aureus* and *Streptococcus uberis* at different concentrations of non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) in cases and controls (Referent group) (n=79 isolates). *Staphylococcus aureus*: HR (95%CI): 0.92 (0.56, 1.49), *P* =0.72. *Streptococcus uberis*: HR (95%CI): 1.23 (0.74, 2.08), *P*=0.45.
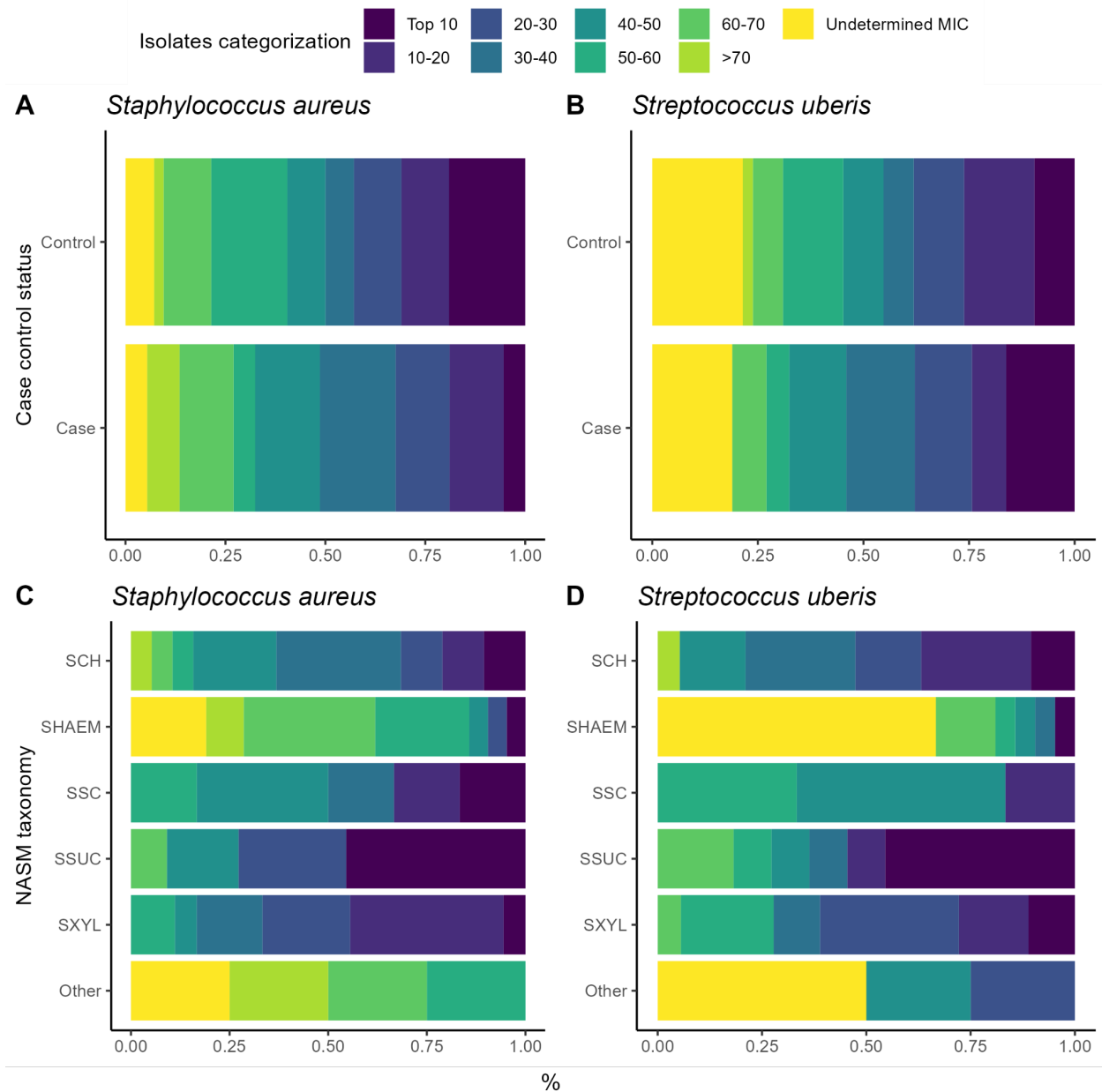
Figure 5.3. Ranking of 79 non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) isolates by minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Streptococcus uberis* harbored from teat apex stratified by case control status (panel A/B) and assigned species (panel C/D) (n=79 isolates). Undetermined MIC: NASM isolates that showed growth of *Staphylococcus aureus* or *Streptococcus uberis* at maximum investigated concentration on media. SCH: *Staphylococcus chromogenes*. SHAEM: *Staphylococcus haemolyticus*. SSC: *Staphylococcus sciuri*. SSUC: *Staphylococcus succinus.* SXYL: *Staphylococcus xylosus/pseudoxylosus.* Other: *Staphylococcus cohnii, Staphylococcus devreseii* and *Staphylococcus equorum.*
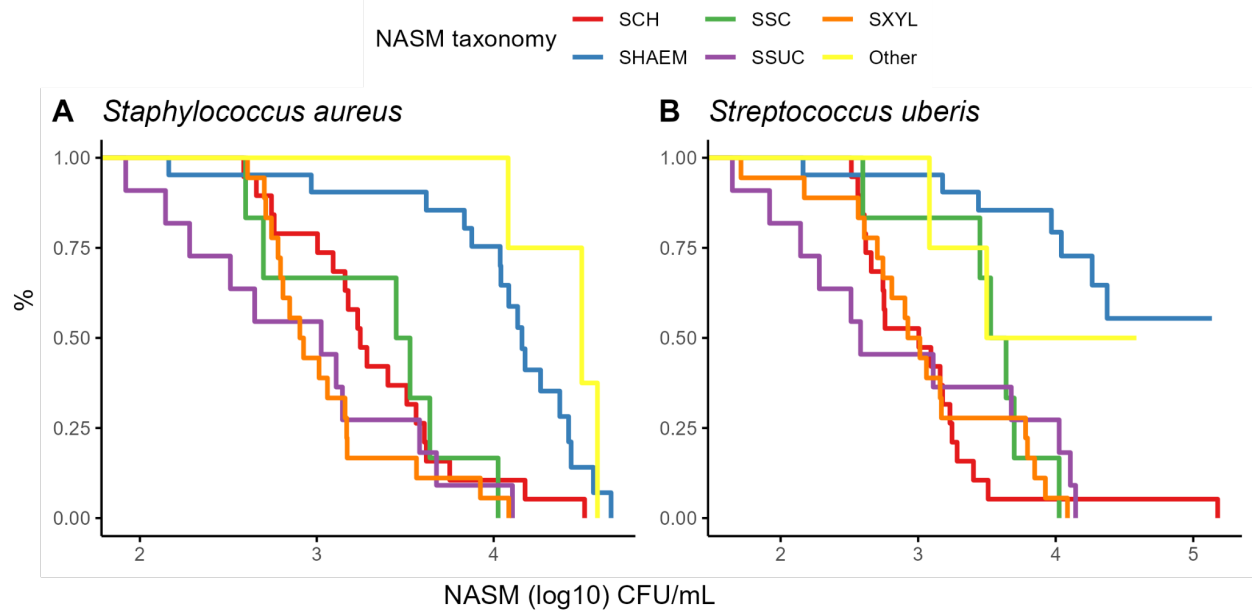
Figure 5.4. Kaplan meier plots showing presence of growth of *Staphylococcus aureus* and *Streptococcus uberis* at different concentrations of non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (NASM) for the different species isolated in this study (n=79 isolates). SCH: *Staphylococcus chromogenes*, SHAEM: *Staphylococcus haemolyticus*, SSC: *Staph sciuri*, SSUC: *Staphylococcus succinus,* SXYL: *Staphylococcus xylosus/pseudoxylosus,* Other: *Staphylococcus cohnii, Staphylococcus devreseii* and *Staphylococcus equorum.* Type III *P-value Staphylococcus aureus* < 0.001. Type III *P-value Streptococcus uberis* < 0.001.

CHAPTER 6: WHOLE GENOME SEQUENCING OF NON-AUREUS STAPHYLOCOCCI AND CLOSELY RELATED MAMMALIICOCCAL SPECIES ISOLATED FROM THE TEAT APEX OF DAIRY COWS RAISED UNDER ORGANIC CERTIFICATION

6.1. SUMMARY

Multiple studies have shown that non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (**NASM**) possess inhibitory activity against mastitis pathogens. In our previous chapter, we discovered an association between the presence of isolates with high *in vitro* inhibitory activity against *Staphylococcus aureus* (**SAU**) on the teat apex and the absence of intramammary infections (**IMI**) by SAU and *Streptococcus* and *Streptococcus*-like organisms (**SSLO**). However, our study did not uncover the potential reasons for the *in vitro* antimicrobial activity of NASM. In addition, only a few studies have investigated the entire genome of NASM. Our first objective was to describe the phylogeny of NASM isolates collected from the teat apex of dairy cows, including the distribution of genes related to antimicrobial peptides (**AMPs**), virulence, and antimicrobial resistance. Our secondary objective was to explore associations between the NASM phylogeny and *in vitro* inhibitory activity of the NASM isolates, as well as the associations with the presence of SAU and SSLO IMI in the cows from which the NASM were isolated. Quarter milk samples from 114 cows in 2 organic dairy farms were taken on a weekly basis for the first 35 days in milk (**DIM**) and submitted for milk culture. Cases were defined as cows that experienced an IMI caused by SAU or *Streptococcus* and *Streptococcus*-like organisms (**SSLO**). Controls were randomly selected from cows that did not have SAU or SSLO IMI. Cases and controls were matched by farm, lactation number, and DIM of IMI diagnosis (within ±7 DIM). Samples from each cow were aerobically cultured and the taxonomy of resultant isolates was confirmed through Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass spectrometry. Isolates classified as NASM were subjected to whole genome sequencing using Illumina MISEQ. Maximum likelihood trees were built by the utilization of

Snippy and IQ-TREE. SPAdes was used for genome assembly. Species-level taxonomy was confirmed using GTDB-Tk. The genomes were annotated for the presence of AMP gene clusters using BAGEL 4, virulence genes were identified using BLAST+, and antimicrobial resistance genes were detected using ABRicate. Logistic regression was used to investigate the association between IMI status, and the presence of AMP gene clusters. Fisher test was used to explore the presence of AMP gene clusters across different NASM species and clades within species. All isolates harbored at least one AMP associated gene in their genome. Except for *Staphylococcus succinus*, NASM in vitro antimicrobial activity was not associated with clade membership. A high virulence gene prevalence was observed in SAU, with NASM species generally showing a lower species-dependent prevalence. This study improves our understanding of the antimicrobial activity and virulence potential of NASM isolates harbored from teat apices of organic dairy cows.

6.2. INTRODUCTION

Non-*aureus Staphylococci* and related *Mammaliicoccal* species (**NASM**) are the most prevalent microorganisms that lead to intramammary infections (**IMI**) on dairy farms (De Buck et al., 2021; Peña-Mosca et al., 2023b). The role of these microorganisms on udder health has been a matter of great debate in recent years (De Buck et al., 2021). Most studies have shown that NASM IMI are associated with a mild increase in somatic cell count (**SCC**) (Paradis et al., 2010; Piepers et al., 2010; Fry et al., 2014; Tomazi et al., 2015; Nyman et al., 2018; Valckenier et al., 2020; Peña-Mosca et al., 2023a). In addition, a few studies have shown increased milk production in cows in which NASM were identified in milk samples (Compton et al., 2007a; Piepers et al., 2010, 2013), while other studies found no association with milk production

(Paradis et al., 2010; Tomazi et al., 2015; Heikkilä et al., 2018; Nyman et al., 2018; Valckenier et al., 2020). Many of these discrepancies could be attributed to the divergent epidemiology, antimicrobial activity, and virulence profiles across NASM species and strains (De Buck et al., 2021).

In a meta-analysis, it was demonstrated that the infusion of NASM into the mammary gland was related to a lower risk of colonization of the udder by major mastitis pathogens (Reyher et al., 2012). This protective effect against major mastitis pathogens has been attributed to the presence of *in vitro* inhibitory activity against these microorganisms in multiple studies (De Vliegher et al., 2004c; Carson et al., 2017; Toledo-Silva et al., 2022). In the previous chapter (chapter 5), we described that the presence of isolates with high *in vitro* inhibitory activity against *Staphylococcus aureus* (**SAU**) on the teat apex was associated with a reduced likelihood of major mastitis pathogen IMI. These results suggested that colonization of the teat apices by NASM strains with high *in vitro* antimicrobial activity could play a role in mastitis control. However, our previous study did not explore potential mechanisms behind the observed protective association between NASM and SAU.

One potential explanation for the *in vitro* antimicrobial activities described in the previou chapter is the ability of NASM to produce antimicrobial peptides (**AMPs**) (Nascimento et al., 2005; Braem et al., 2014; Carson et al., 2017), commonly referred to as bacteriocins or *Staphylococcins* (de Freire Bastos et al., 2020; Newstead et al., 2020). Although not yet commercially available, AMPs represent a possible alternative to antibiotics that could be used for mastitis control on dairy farms (Nascimento et al., 2005; Braem et al., 2014; Carson et al., 2017). While the prevalence of AMPs have been previously investigated in NASM, more

research is needed to identify molecules that could be targeted for the development of post-biotic products that could be used for mastitis control. Lastly, although there has been significant expansion in our understanding of NASM epidemiology in recent years, the number of studies that have comprehensively investigated the entire genome of NASM are still limited (Naushad et al., 2016, 2019; De Buck et al., 2021; Fergestad et al., 2021). This limits our ability to investigate associations between NASM genotypes, phenotypes, and cow-level udder health outcomes. Therefore, more research is needed to describe the genomic diversity of NASM isolates from dairy cows, including important phenotypic drivers such as genes related to virulence and antimicrobial resistance.

Our first objective was to describe the phylogeny of NASM isolates collected from the teat apex of dairy cows, including the distribution of genes related to AMPs, virulence, and antimicrobial resistance. Our secondary objective was to explore associations between the NASM phylogeny and *in vitro* inhibitory activity of the NASM isolates, as well as the associations with the presence of SAU and SSLO IMI in the cows from which the NASM were isolated.

6.3. MATERIALS AND METHODS

This manuscript was prepared following the guidelines outlined in the Strengthening the Reporting of Observational Studies in Epidemiology - Veterinary (**STROBE-Vet**) Statement (Sargeant et al. in 2016). All study activities were approved by the University of Minnesota Institutional Animal Care and Use Committee (**IACUC**) (Protocol Number: 1807: 36109A), Colorado State University IACUC (Protocol number: 1442), and Texas Tech University IACUC

(Protocol number: 18068-10). Laboratory activities were approved by the Institutional Biosafety Committee (IBC) protocol: 2205-40001H.

6.3.1. Inclusion criteria

This study included cows from USDA-certified organic dairy farms across various regions in the United States, with different herd sizes. Farm selection was based on willingness to participate and proximity to collaborating universities. Despite our initial intention to include farms with electronic records, we added a smaller farm without such records to maintain a balanced representation of both small and large farms. The research was part of a broader investigation into the udder microbiome and udder health (Dean et al., 2021). We initially enrolled cows from five organic dairy farms but, for this report, we focused on data from just two farms due to practical constraints related to research staff availability. The analysis was conducted on samples collected from 114 cows, comprising 93 from Farm A (collected between August 2019 and January 2020) and 21 from Farm B (collected between February and July 2021).

6.3.2. Implementation of case control study

In this study, cases were cows that had an IMI caused by SAU or SSLO within the first 35 days in milk (**DIM**). Controls were randomly selected from the enrolled cows at Farms A and B that did not have IMI caused by these microorganisms during the first 35 DIM. It is important to note that both cases and controls may have had IMIs caused by other microorganisms in their milk samples, such as gram-negative bacteria or NASM. However, these additional infections were not considered when choosing cases and controls or in the subsequent analysis. The cases

and controls were matched based on the farm, lactation number, and the number of days relative to calving when the first SAU or SSLO diagnosis was made (within ± 7 DIM). For each selected cow, one teat apex sample from the week before acquiring a new IMI was processed using the methodology outlined below. Three cows did not have a teat apex sample available from the week before a new IMI. Hence for these animals two samples were processed, resulting in a total of 71 samples collected from 68 cows (comprising 34 cases and 34 controls).

6.3.3. Teat apex sample collection

From eight weeks before calving to five weeks following calving, all enrolled cows underwent weekly sampling. Teats were first visually inspected, and any material that was found was cleaned off with a paper towel. The distal third of each of the four teats was then carefully swabbed with a single piece of sterile, pre-moistened gauze while wearing a fresh set of disposable gloves. The gauze was added to individual tubes that had been pre-loaded with a 50% glycerol solution after sampling. Gauze samples were transported in a cooler with ice and stored at -80 °C after arrival to the research facilities. Postpartum samples were collected before applying pre-dipping disinfection solutions to the teat and before collection of milk samples.

6.3.4. Teat apex culture and species identification

The methods for culture and species-level identification were adapted from protocols utilized in the Laboratory for Udder Health at the University of Minnesota to process towel samples (Rowe et al., 2019). Before sample processing and in an effort to reduce the possibility of batch effects, sample IDs were randomly sorted using Excel (Microsoft Corporation, Redmond). In accordance with this random order, isolates were selected for each sample

processing date in batches of 10 isolates. Gauze samples were thawed at 4 °C overnight and then transferred into 50 mL round-bottom falcon tubes (Corning, Inc.). Afterward, 5mL of Phosphate-Buffered Saline (PBS) (Gibco, Termofisher Scientific) was added to reduce viscosity. Samples were homogenized carefully by turning tubes upside down twice and vortexing for 10 seconds at a low speed. Samples were allowed to stand for 10 minutes and the homogenization process was repeated. Following homogenization, a 1 in 10 serial dilution was done using PBS with a dilution scheme ranging from 10^{-1} to 10^{-7} (Gibco, Termofisher scientific). A 100 uL aliquot from each diluted sample was inoculated onto Mannitol salt agar (Oxoid). Plates were then incubated in aerobic conditions for 24 hours at 37 °C. Following incubation, bacterial groups were visually assessed and counted from the dilution plate with the optimal number of colonies (25 to 250 per plate). Isolates were then picked from the plate using a sterile calibrated loop (Thermofisher Scientific), streaked onto blood agar (Hardy Diagnostics), and incubated for 24 hours at 37 °C. After achieving pure growth, the identity of representative colonies was studied further using a Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometer (MS) (Microflex; BrukerDaltonics Inc.) (Jahan et al., 2021). Each isolate's mass spectra profile was compared to the Biotyper reference library. Confidence scores were employed to assign genus and species-level classifications (1.8 and 2.0, respectively) (Jahan et al., 2021). Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species isolates were then preserved at -80 °C using commercial cryovials (Hardy Diagnostics) foruntil further analysis. NASM species-level taxonomy was further investigated through whole genome sequencing, as reported below

6.3.5. Determination of in vitro inhibitory activity against *Staphylococcus aureus* and *Streptococcus uberis*

The methods employed in this manuscript were adapted from those previously described (Toledo-Silva et al., 2022). Excel (Microsoft Corporation) was utilized to randomly sort isolates on the first day of each week. Subsequently, isolates previously identified as NASM using MALDI-TOF MS were cultured on blood agar plates (Hardy Diagnostics) for 24 hours. If mixed growth was observed, isolates were re-plated. On the second day, a single colony was selected using a 10 µL disposable loop and cultivated on brain heart infusion (**BHI;** Research Products International Corporation) media for 18 to 20 hours. The following day, 1 in 10 serial dilution solutions were generated in a 96 well plate (Thermo Fisher Scientific) using sterile PBS. The bacterial count (colony forming units (**CFU**)/mL) was determined by plating 100 µL of the serial dilutions on blood agar plates and incubating them aerobically at 37 °C for 18 to 20 hours. Inside Falcon round-bottom polypropylene tubes (Corning Inc.), 100 microliters of bacterial suspensions containing NASM isolates at various dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) were added to 10 mL of 2% Tryptic soy agar (TSA; Oxoid). Before adding 2% TSA, the temperature was checked with a laboratory digital thermometer (Thermco). The media were not employed until the temperature reached 50 °C (Toledo-Silva et al., 2022). The tubes were thoroughly mixed and then transferred onto 4% TSA plates that had been preheated to 37 °C. After aerobic incubation for 18 to 20 hours, 10 µL of bacterial suspensions containing approximately 10,000 CFU of SAU (American type culture collection (**ATCC**) 25923) and SUB (ATCC 19436) were transferred onto adjacent spots on the plates containing varying concentrations of NASM isolates, followed by an additional incubation of 18 to 20 hours. For all batches, we included

negative controls by placing a 2% TSA agar plate with 100 µL of only BHI (omitting NASM isolates) during incubation. Furthermore, an additional 10 µL of PBS was placed on the surface of the media on every plate, acting as an extra negative control to verify the absence of PBS contamination. The presence or absence of growth of SAU and SUB was determined by visually inspecting each plate and comparing them to the negative control plates in the respective batches. The minimum inhibitory concentration (**MIC**) was defined as the lowest concentration of NASM (CFU/mL) in the 2% TSA top layer that could inhibit the growth of SAU or SUB. Thereafter, isolates were ranked based on their MIC (ranging from 1 to 79) against SAU or SUB and categorized as either "top 10" NASM isolates with the lowest MIC or not.

6.3.6. Milk sample collection

Following recommendations from the National Mastitis Council (NMC, 2017), quarter milk samples were taken weekly for the first five weeks following calving. Milk sampling was performed following collection of teat apex samples and dipping the teats on a disinfectant solution. Three to four streams of milk were discarded from each quarter, and the teat apex of each quarter was cleaned with a cotton soaked in 70% ethanol. The milk samples were collected starting with the right rear quarter and proceeding in a clockwise sequence. Each quarter's milk was collected into individual 60 mL plastic vials (Capitol Vials, Termo Fisher). Samples were placed on ice immediately after collection and stored in a freezer at -20 °C until further processing.

6.3.7. Milk pooling

The following methodology was used to pool quarter milk samples into composite samples (Peña-Mosca et al., 2023b). First, overnight thawing at 4 °C was done on milk samples. Then, quarter-sized milk samples were homogenized by turning them over twice while upside down. Then, under a laminar hood, 2 mL were drawn out of each plastic vial and dispensed into a single vial. The University of Minnesota's Udder Health Laboratory in St. Paul, Minnesota, USA, received the lab-pooled composite samples and processed them for milk culture.

6.3.8. Milk culture

Using a cotton swab, about 100 μL of milk was applied to Columbia CNA agar with 5% sheep blood and MacConkey agar. The agar plates were then incubated in aerobic conditions at 37 °C for a total of 42 to 48 hours. A trained technician inspected the plates to check for the growth of distinct isolates at both the 18-24 hour and 42-48 hour marks. If more than three different isolates were found in a sample, it was classified as contaminated and excluded from further analysis (Dean et al., 2022). To determine the taxonomic classification of the cultured isolates, we employed MALDI-TOF MS, following the same methodology used for teat-apex samples. An IMI was defined as a composite sample containing one or more than one colony forming units (10 CFU/mL) of any cultured isolates. Microorganisms were grouped according to their morphological and epidemiological characteristics in different bacterial groups: SAU, NASM (members from *Staphylococcus* genus that were not SAU), SSLO which include members from *Streptococcus* genus, as well as *Streptococcus*-like organisms (e.g., *Enterococcus* spp., *Aerococcus* spp., *Lactococcus* spp. and *Micrococcus* spp.), gram negative microorganisms (e.g., *Escherichia* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Enterobacter*

spp., *Pantoea* spp.) and others (microorganisms that did not belong to any of the previous groups).

6.3.9. DNA extraction, library preparation and whole genome sequencing

Isolates were allowed to grow on BHI for 24 hours, centrifuged at 10,000 rpm for 5 minutes, and the pellet was collected in 2mL sterile polypropylene microcentrifuge tubes (Thermo Fisher Scientific). Following this, DNA extraction was performed utilizing commercial kits (Qiagen's DNeasy 96 Blood & Tissue Kit, Qiagen). Amount and quality of extracted DNA was evaluated using PicoGreen fluorometer and NanoDrop spectrophotometer (Thermo Fisher Scientific). Library preparation was performed using the DNA Prep Sample Preparation Kit (Illumina Inc.). Resultant DNA was subjected to paired-end sequencing (2x300) utilizing the Illumina MISEQ platform aiming for 50x coverage of *Staphylococcus* spp. genome.

6.3.10. Bioinformatics

Quality control of raw sequencing data was performed using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016). Trimmomatic was utilized to trim low quality and adapters from raw reads (Bolger et al., 2014). MultiQC was then utilized to summarize the QC metrics during the filtering and trimming process. Genomes were assembled utilizing SPAdes version 3.15.5 (Bankevich et al., 2012). Quality of assemblies was evaluated using Quast version 5.2.0 (Gurevich et al., 2013). The species identification of isolates was performed utilizing GTDB-Tk version 1.7.0 (Chaumeil et al., 2020) and GTDB release 202 (Parks et al., 2022) as implemented in KBase (Arkin et al., 2018). ABRIcate (Seemann, 2023a) with default settings was utilized for identification of antimicrobial resistance genes (**ARGs**), using MEGARes v2 as the reference

database (Doster et al., 2020). To identify virulence factors (**VFs**), we utilized BLAST+

(Camacho et al., 2009) and a custom comprehensive database (Naushad et al., 2019), previously

proven effective for identification of virulence genes in *Staphylococcus* genomes (Naushad et al.,

2019; Fergestad et al., 2021). Only BLAST+ results with an e-value of less than or equal to 10^{e-5}

(Choudhuri, 2014) and amino acid identity of greater than or equal to 35% (Rost, 1999) were

considered for analysis. The BAGEL4 online server with default settings was used to identify

gene clusters associated with the production of AMPs (van Heel et al., 2018). Snippy was used

with default settings (i.e., minimum coverage of 10x and a minimum variant fraction of 90%) to

identify core single nucleotide polymorphisms (Seemann, 2023b) by aligning trimmed reads

from each of the isolates onto reference genomes obtained from Genbank. The following

reference genomes were used for each NASM species: *Staphylococcus chromogenes* (**SCH**):

GenBank accession number: GCA_002994305.1, *Staphylococcus haemolyticus* (**SHAEM**):

GenBank accession number: GCA_001611955.1, *Staphylococcus sciuri* (**SSC**): GenBank

accession number: GCA_002209165.2, *Staphylococcus succinus* (**SSUC**): GenBank accession

number: GCA_001902315.1, *Staphylococcus xylosus* (**SXYL**) and *Staphylococccus*

pseudoxylosus (**SPXYL**): GenBank accession number: GCA_000709415.1. As a result of the

limited availability of only one or two genomes for these species, SNP calling was not performed

for *Staphylococcus devrieseii* (**SDEV**), *Staphylococcus equorum* (**SEQ**)*,* and *Staphylococcus*

cohnii (**SCON**). The generated Snippy core files were used to build a maximum likelihood tree

using IQ-TREE, with 1,000 bootstrap replicates to assess the robustness of the phylogenetic tree

(Nguyen et al., 2015). Trees were visualized and annotated using iTOL (Letunic and Bork,

2021).

6.3.11. Statistical analysis

All analyses were performed using R 4.3.2. Because NASM species were strongly associated with *in vitro* inhibitory activity and presence of AMP gene clusters, all statistical analyses were stratified by NASM species (SCH, SHAEM, SSC, SSUC, SXYL, SPXYL, Others (SCON, SEQ, SDEV). A clade, also known as a monophyletic group, was defined as a group of genomes on a phylogenetic tree that included their most recent shared ancestor and their descendants (Kapli et al., 2020). Only AMP gene clusters that were both present and absent in at least 2 isolates within a given species were investigated using regression analysis. For modeling, the association between IMI status, clade membership and the presence of AMP gene clusters was investigated using logistic regression. The presence of AMP gene clusters across different NASM species, and clades within species, was compared using the Fisher exact test. This approach was chosen because, in several cases, gene clusters were found exclusively in a single clade or species. The association between presence of AMP gene clusters, clade membership, and the MIC of NASM against SAU and SUB for each NASM species was investigated using linear regression.

6.4. RESULTS

6.4.1. Descriptive characteristics

This study examined two farms in Minnesota (A) and Colorado (B) with varying herd sizes and housing systems. Both farms met organic regulations by having cows consume at least 30% of their diet from pasture, when possible. On Farm A, all sampled cows included in this study (14/14) were in their first lactation, while in Farm B, roughly half (43/93) were in their

first lactation, and half (50/93) were transitioning to their second lactation. The case control study from which isolates were obtained included 68 animals from these two farms (34 cases and 34 controls). Fifteen of the cases had a SAU-IMI, 15 had a SSLO-IMI and 4 had an IMI caused by both microorganisms. In the first postpartum composite milk sample, we discovered that 64.7% of the cases were already positive for SAU or SSLO IMI. Teat apex samples from cases and controls were collected on similar average days relative to calving (mean [SD]; Controls: -2.8 [15.2] vs Cases: -1.2 [16.5]). From the 71 teat apex samples cultured for NASM, a total of 275 isolates were collected and submitted for MALDI-TOF MS. A high proportion of these isolates were identified as *Bacillus* spp. (61.8% [170/275]). Non-*aureus Staphylococci* and closely related *Mammaliicoccal* species were identified in 30.2% (83/275) of the isolates. After reconfirming species identification through whole genome sequencing, the most common species in the NASM group were SCH (8.0% [22/275]) and SHAEM (7.6% [21/275]).

6.4.2. Assembly quality

Across the assembled genomes, an average ± SD of 48.4 ± 137 contigs were identified. The mean ± SD genome size was 2,610,000 ± 276,000, with the N50 value averaging 585,000 ± 485,000 across all genomes. The longest contig exhibited an average size of 883,000 ± 367,000 across all genomes.

6.4.3. Phylogeny and antimicrobial activity

Five phylogenetic trees were constructed representing six different NASM species (SCH, SHAEM, SSC, SSUC, SXYL/SPXYL). The assigned NASM taxonomy showed a strong association with the presence of various AMP gene clusters. For example, we observed that AIP

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class I gene clusters (NCBI accession number: WP_001093929.1) were present in all isolates identified as SCH and SHAEM, as well as in half of those identified as SSC, but were absent in SSUC, PXYL, and SPSXYL (*P*<0.001). Similarly, putative bacteriocin 193.2 (Genbank accession number: AJ002203.2) gene clusters were highly prevalent in SXYL or SEQ, but were not detected in other NASM species (*P*<0.001).

Staphylococcus chromogenes

A total of 21 isolates were identified as SCH (**Figure 6.1**), 12 of which were obtained from the teat apex of control cows (i.e., without SAU or SSLO IMI), and 9 from cases (i.e., with SAU or SSLO IMI). Furthermore, 20 out of 21 isolates were found in cows from farm A, with only 1 cultured from the teat apex of a cow from farm B.

The mean (SD) MIC (CFU/mL) of SCH on SAU and SUB was 3.32 (0.50) and 3.06 (0.60), with 10.5% (2/19) and 10.5% (2/19) of the isolates classified as "top 10" with lowest MIC, respectively. Genes encoding AIP class I production were identified in 76.2% (16/21) of isolates, while auto-induced peptides class II (NCBI accession number: WP_001094921.1) were found in 23.8% (5/21) of isolates. Sactipeptides (Interpro accession number: PF04055) and subtilosin A (NCBI accession number: NP_391616.1) gene clusters were highly prevalent in SCH, identified in 100% (21/21) and 61.9% (13/21) of isolates, respectively. Lanthipeptides class II (Interpro accession number: PF05147) gene clusters were identified in 1 out of the 21 isolates (4.8%). Gene clusters associated with the production of AIP class I were present in (5/9) 55.6% of SCH isolates obtained from teat apices of cases and 91.7% of those obtained from controls (11/12) (*P*=0.08). AIP class II gene clusters were rarely identified in controls 8.3%

(1/12) but highly prevalent in cases 44.9% (4/9) (*P*=0.08). Subtilosin A gene clusters were present in 5 out of 12 isolates (41.7%) in controls and 3 out of 9 isolates (33.3%) in cases, respectively (*P*=0.70). Nonetheless, presence of the aforementioned AMP gene clusters in SCH genomes was not related to the presence of *in vitro* antimicrobial activity (i.e., MIC against SAU or SUB) (*P* > 0.05 for all linear regression models).

Phylogenetic analysis of the 21 SCH genomes revealed two major clades, with one clade containing just two isolates, both from the same cow (SCH7 and SCH14 from cow 46, **Figure 6.1**). The largest subclade contained 9 isolates (SCH1, SCH3, SCH5, SCH8, SCH15, SCH16, SCH17, SCH18, SCH20), six of which originated in controls and three in cases. The genomes within this clade were characterized by the presence of subtilosin A gene clusters, which were found in all isolates from this clade (100%, 9/9), but had a lower prevalence in other SCH isolates (33%, 4/12) (P < 0.001). Additionally, AIP class I was identified in the genomes of all SCH isolates within this clade (100%, 9/9), while it was present in around half of the other SCH isolates (58.3%, 7/12) (*P*=0.045). Notably, AIP class II was not identified in this clade but was found in 41.7% of the other SCH isolates (*P*=0.045). This clade also contained the only 2 SCH isolates classified as "top 10" with highest antimicrobial activity. However, we did not find evidence of a difference in the MIC (CFU/mL) against SAU or SUB when comparing isolates in this clade with isolates in the other clades (SAU: estimate [95%CI]: 0.05 [-0.45, 0.56]), *P*=0.82; SUB: -0.35 [-0.93, 0.22], *P*=0.51). In addition, these 21 isolates were obtained from 16 cows, with 5 cows carrying 2 SCH isolates each. In certain instances, the two isolates from a single cow exhibited very minor degrees of divergence and were grouped within the same clade (e.g., cow 461: SCH7 and SCH14; cow 863: SCH3 and SCH18; cow 1086: SCH2 and SCH21).

However, in other cases, SCH isolates from distinct clades were isolated from the same cow and on the same day (e.g., cow 87835: SCH5 and SCH11; cow 89386: SCH5 and SCH15), indicating the presence of different strains within the SCH population colonizing the teat apex.

Staphylococcus haemolyticus

A total of 19 isolates were identified as SHAEM (**Figure 6.2**). Out of these isolates, 10 were obtained from the teat apex of control cows, and 9 were from cases. Eighteen out of 19 isolates were found in cows from farm A, with only 1 isolate detected in a cow from farm B. We were unable to determine the MIC against SAU or SUB in a considerable number of the isolates since they did not show any evidence of inhibitory activity at the maximum studied concentration (21.1%[4/19] for SAU; 68.4% [13/19] for SUB). For isolates with a determinable MIC, it was 3.95 (0.60) and 4.06 (0.72). None of the isolates classified as SHAEM were found to be among the "top 10" in terms of inhibitory activity against these target microorganisms. Genes encoding AIPs class I and sactipeptides gene clusters were identified in 100% (19/19) of the isolates. Lanthipeptides class IV gene clusters were identified in 1 out of the 19 isolates (5.3%).

The 19 SHAEM isolates were grouped into 2 distinct clades, with one clade comprising just two isolates, as shown in **Figure 6.2**. The largest subclade contained 10 isolates: SHAEM2, SHAEM3, SHAEM4, SHAEM7, SHAEM9, SHAEM13, SHAEM14, SHAEM16, SHAEM18, SHAEM19. Half of these isolates were obtained from controls (5/10) and half from cases (5/10). Similar to other SHAEM isolates, isolates from this clade did not show signs of inhibitory activity with a considerable proportion of isolates being unable to inhibit the growth of SAU (30.0% [3/10]) or SUB (90.0% [9/10]), or classified above the median MIC (i.e., ranking MIC

above 40). Interestingly, a small subclade containing 2 isolates (SHAEM 8 and SHAEM10)

harbored from controls, encompassed isolates obtained from cows in different farms and

exhibiting different inhibitory activity against SAU or SUB. We did not investigate potential

associations with genes associated with AMP production because they either had low prevalence

or were present in all SHAEM isolates.

Staphylococcus sciuri

Six isolates were identified as *Staphylococcus sciuri* (**Figure 6.3**)*.* Half of these isolates

were cultured from controls (3/6) and half from cases (3/6). In addition, 4 of these isolates were

obtained from cows in farm A and 2 from cows in farm B. The genomes of these isolates

harbored genes that encoded AIPs class I (controls: 66.7% [2/3], cases: 33.3% [1/3]),

sactipeptides (present in all isolates) and putative bacteriocin 194.2 (Genbank accession number:

AJ002203.2) (controls: 33.3% [1/3], cases: 66.7% [2/3]). No associations were found between

presence of these genes, which are related to the production of AMPs, and the MIC against SAU

or SUB (*P*>0.05 for all linear regression models).

Phylogenetic analysis showed that isolates were grouped in 2 distinct clades, one

including isolates from farm A and the other one isolates from farm B (**Figure 6.3**). Isolates from

farm A formed two different subclades, one including two isolates obtained from cases (SSC1

and SSC2) and the other from controls (SSCI5 and SSCI6). Putative bacteriocin 194.2 was

identified in all isolates isolated from cows in farm A (4/4) but not identified in those from farm

B (0/2) (*P*=0.08). No differences were encountered in the MIC against SAU or SUB across these

clades (*P*=0.92 and *P*=0.21, respectively).

Staphylococcus succinus

A total of 12 isolates were identified as SSUC (**Figure 6.4**). Fifty percent (6/12) were isolated from cases, and the remaining fifty percent (6/12) from controls. Furthermore, all SSUC isolates were found in cows from farm A. The average MIC of SSUC on SAU and SUB was 2.92 (0.69) and 2.92 (0.93), respectively. Almost half, 45.5% (5/11) of the isolates were classified as "top 10" with lowest MIC. Genes encoding AIPs class II and Sactipeptides were identified in 100% (19/19) of the isolates. Carnolysins were identified in 1 out of the 12 isolates (8.3%).

Staphylococcus succinus isolates were grouped in multiple clades (**Figure 6.4**). One of these clades contained isolates SSUC1, SSUC2, SSUC3, SSUC4, SSUC5, SSUC10 and SSUC12, and the MICs of these isolates were lower than SSUC isolates in other clades (estimate [95%CI]: MIC SAU: -1.06 [-1.66, -0.46], *P*=0.003; MIC SUB: -1.06 [-2.14, 0.02], *P*=0.05). This clade also contained all of the SSUC isolates classified as "top 10" with lowest MIC against both SAU and SUB. Lastly, an interesting finding was that there were 2 cows from which multiple isolates were recovered (cow 94706: SSUC1, SSUC7 and SSUC12; cow 1333: SSUC6 and SSUC10), and these isolates were distributed across distinct subclades, suggesting the presence of distinct strains within the teat apex of a given cow in the same sampling point. Associations between the presence of genes related to the production of AMPs were not further investigated because they showed a low prevalence or were present in all SSUC isolates.

Staphylococcus xylosus and *Staphylococcus pseudoxylosus*

We identified 13 isolates as SXYL and 5 as SPXYL (**Figure 6.5**). Among SXYL isolates, 53.8% [7/13] were isolated from controls and 46.2% [6/13] from cases. For PXYL, 60.0% [3/5]

and 40.0% [2/5] of SPXYL isolates were obtained from controls and cases, respectively. The

majority of SXYL and SPXYL isolates were identified in cows from farm B (92.3% [12/13] and

80.0% [4/5], respectively), with only 1 isolate from each of these species isolated from cows in

farm A (SXYL: 7.7% [1/13] and SPXYL: 20.0% [1/5]).

The phylogenetic tree showed that isolates could be divided into 3 distinct clades, two of

which contained isolates from SXYL (SXYL clade A: SXYL1, SXYL2, SXYL4, SXYL10,

SXYL11, SXYL13, SXYL14; SXYL clade B: other SXYL isolates) and the other of which

contained isolates classified as PSXYL. The MIC (CFU/mL) against SAU was 3.14 (0.18), 2.98

(0.17), and 3.06 (0.20) for isolates in SXYL clade A and B and for those identified as SPXYL,

respectively (Type III *P-value* = 0.81). In addition, for isolates belonging to SXYL clades A and

B as well as those recognized as SPXYL, the MIC against SUB was 3.12 (0.26), 2.97 (0.28), and

3.06 (0.30), respectively (Type III *P-value* = 0.92). Sactipeptides and subtilosin A were present

in all SXYL and SPXYL genomes. On the other hand, AIPs class II were present in 53.8% (7/13)

of the SXYL isolates (clade A: 33.3% [2/6]; clade B: 71.4% [5/7] and 100% [5/5] of the PSXYL

isolates) (*P*=0.07). In addition, AIPs class III (NCBI accession number: WP_000735197.1) were

highly prevalent in SXYL clade A (66.7% [4/6]); showed a low prevalence in SXYL clade B

(14.3% [1/7]); and were absent in SPXYL (*P*=0.03). Putative bacteriocin 193.2 genes were

identified in all isolates from SXYL clade B (100%, 6/6), had a lower prevalence in SXYL clade

A (14.3%, 1/7), and were not found in SPXYL isolates (*P*<0.001). None of these genes

associated with production of AMPs were associated with MIC against SAU or SUB (*P*>0.05 for

all linear regression models).

6.4.4. Genetic determinants of virulence

Adherence

A total of 37 genes involved in adherence were investigated in this study, from which 30 were detected (81.1%) (**Figure 6.6**). Adherence genes were highly prevalent in SAU and nearly all the genes were present in at least 1 SAU isolate (81.1% [31/37]). Conversely, these genes showed a lower presence in NASM (% genes identified in different NASM: SCH: 51.4% [19/37] SHAE: 56.8% [21/37], SSC: 54.1% [20/37], SSUC 37.8% [14/37], SXYL: 59.5% [22/37], SPXYL: 45.9% [17/37]). Some of these genes (e.g., accumulation associated protein [**aap**], biofilm-associated surface protein [**bap**], autolysin [**atl**], clumping factor class B [**clfB**], cell wall surface anchor family protein class G [**sasG**], serin rich adhesion for platelets [**sraP**], Ser-Asp rich fibrinogen-binding bone sialoprotein-binding proteins [**sdrC**, **sdrD**, **sdrE**, **sdrF**, **sdrH**, **sdrI**] and cell wall anchored protein class A [**uafA**]) were present in nearly all the genomes of the studied NASM species. In addition, fibrinogen-binding protein class A and B (**fnbA**, **fnbB**) were highly prevalent in SCH (19/21 [90.4%]), SXYL (12/13 [92.3%]) and SPXYL (5/5 [100%]). Intercellular adhesion proteins (**ica**) were highly prevalent in isolates identified as SCH (icaA only; 64.3% [9/21]) and SSC (icA-D; 6/6 [100%]), but showed a lower prevalence in other NASM species.

Exoenzymes

This study included the investigation of a total of 22 exoenzymes (**Figure 6.6**). In SAU, we identified 17 genes responsible for exoenzyme production (77.3%). In contrast, NASM contained a lower number of exoenzymes genes in their genomes (SCH: 27.3% [6/22], SHAEM:

14.2% [4/22], SSC: 27.3% [6/22], SSUC: 40.9% [9/22], SXYL: 36.4% [8/22], SPXYL: 31.8% [7/22]) with various types of exoenzymes present across different species. Among these genes, nucleases (**nuc**) were identified in all NASM isolates (81/81 [100%]). Aureolysin (**aur**) and nucleases was identified in all NASM species except for SHAEM (61/62 [98.4%]). Glycerol ester hydrolase (**geh**) and lipases (**lip**) genes were detected in all the isolates identified as SCH, SHAEM, SXYL and SPXYL, but showed a low prevalence in SSC (0/6 [0%]) and SSUC (3/12 [25.0%] for both genes). Adenosine synthase A (**adsA**) was identified in all SCH, SHAEM and SDEV isolates, but was not present in other NASM species. Various serine proteases (**sp, ssp**) were detected among distinct NASM. For instance, splA, splC, splE, and sspA were found in all isolates categorized as SSUC, while splB, splD, and sspA were present in isolates classified as SSC. Isolates belonging to SXYL and SPXYL contained sspE and sspA.

Immune evasion

This study looked into a total of 35 genes associated with bacterial immune evasion (**Figure 6.7**). Most immune evasion genes were present in SAU (97.1% [34/35]), but showed a lower presence in NASM genomes (% genes identified in different NASM: SCH: 85.7% [30/35], SHAEM: 51.4% [18/35], SSC: 22.9% [8/35]; SSUC: 11.4% [4/35], SXYL: 80.0% [28/35] and SPXYL: 11.8% [4/35]). Capsular gene (**cap**) 5P, cap8P and cap5J were identified in nearly all NASM isolates (81/81 [100%], 81/81 [100%] and 80/81 [98.8%], respectively). In addition, cap5H was identified in all SSUC isolates, and in SPXYL, but showed a low prevalence in SCH (7/21 [33.3%]) and *SSC* (1/6 [16.7%]). On the other hand, capsular genes cap5M-P and cap8M-P were highly prevalent in SCH. Staphylococcal binding immunoglobulin protein (**sbn**) and

staphylococcal complement inhibitor (**scn**) were highly prevalent in SCH (20/21 [95.2%] and 18/21 [85.7%], respectively, but not present in other NASM species.

Iron uptake and metabolism

A total of 29 genes involved in iron uptake and metabolism were investigated (**Figure 6.7**). Not surprisingly, SAU showed a high prevalence for all the genes associated with iron uptake and metabolism (100% of the genes were identified in at least one SAU genome [29/29]), while the number of genes detected in at least 1 isolate of different NASM species was substantially lower (SCH: 48.3% [14/29], SHAEM: 55.2% [16/29], SSC: 58.6% [17/29], SSUC: 37.9% [11/29], SXYL: 34.5% [10/29], SPXYL: 37.9% [11/29]). Some of these genes such as FecCD iron compound ABC transporter permease family proteins (**htsA**, **htsB**, **htsC**), staphylococcal iron regulated class B (**sirB**), and staphylococcal iron regulated class C (**sirC**) were present in all the isolates. Other highly prevalent genes were staphyloferrin A biosynthesis proteins (**sfaA**, **sfaB**, **sfaC**); however, these genes were not identified in SSC isolates. Other genes showed a lower prevalence as depicted in **Figure 6.8**.

Enterotoxins and Staphylococcal exotoxins

A total of 87 genes related to the production of enterotoxins and staphylococcal exotoxins were investigated, with 59 distinct genes identified in *Staphylococcus* spp. (**Figure 6.8**). Interestingly, genes that encode the production of enterotoxins were only identified in SAU. Staphylococcal exotoxins were identified in both SAU and SCH but not in other NASM species.

Other toxins

A total of 36 genes that encode the production of other toxins were identified in NASM genomes (**Figure 6.9**). *Staphylococcus aureus* showed a high number of genes that encode the production of toxins (75.0% [27/36]), while the number of toxin genes in NASM was considerably lower, with a few exceptions. For instance, exfoliative toxin type C (**etc**) was detected in all NASM genomes. Similarly, beta phenol-soluble modulins were highly prevalent in all species except for SCH and SSC. In addition, type VII secretion system proteins (**esa** and **ess**) were detected in some SCH isolates (9/21 [33.3%]) but were absent or showed a considerably lower prevalence in other NASM species.

6.4.5. Genetic determinants of antimicrobial resistance

In this study, we identified the presence of 28 different ARGs (**Figure 6.10**). *Staphylococcus aureus* had 15 of these genes in its genome, whereas each NASM species had between 1 and 6 ARGs in at least one isolate. Ninety-seven percent of the isolates (81/83) showed the presence of at least 1 ARG. The most prevalent ARGs were 23S rRNA methyltransferases (**RLMH**) (53.0% [44/83]), involved in the synthesis of an rRNA methyltransferase related to resistance against lincosamides and macrolides. Another gene that exhibited high prevalence was aminoglycoside O-phosphotransferases (**APH3-PRIME**) (50.6% [42/83]), which encodes an aminoglycoside-modifying enzyme. Additionally, macrolide phosphotransferases (**MPHC**), a gene associated with macrolide resistance, was present in 16.9% (14/83) of the isolates. Other genes, including those linked to penicillin resistance (e.g., class A betalactamases [**blaZ gene**]) or methicillin resistance (e.g., penicillin binding proteins [**mecA**, **mecB**, **mecI**]), demonstrated low prevalence (<10%) among SAU and NASM isolates.

6.5. DISCUSSION

This study focused on improving our understanding of NASM genomes collected from the teat apices of organic dairy cows around parturition and their potential antimicrobial activity against major mastitis pathogens. We discovered that in spite of the varying *in vitro* antimicrobial activity of NASM isolates, all NASM harbored at least 1 AMP in their genome. Interestingly, there was no evidence of an association between the presence of gene clusters related to the production of AMPs and the *in vitro* inhibitory activity against SAU or SUB. One clade within the SSUC group comprised 7 NASM isolates, with the majority demonstrating high *in vitro* antimicrobial activity. However, for other species, no association was found between clade membership and in vitro antimicrobial activity. Multiple species and multiple strains of NASM were present in the same cow on the same sampling date. Lastly, our results showed that although SAU genomes showed a higher prevalence of virulence genes and ARGs, all NASM harbored at least some of these genes, with a distribution that appeared to be in most cases species-dependent.

6.5.1. Genotypic and in vitro antimicrobial activity

This is the first study that investigated both the genotypic and phenotypic antimicrobial activities of NASM on organic dairies. One of the most intriguing observations we made was the ubiquitous presence of gene clusters associated with the production of at least one AMP in all examined genomes. This finding contradicts a prior study conducted with isolates from conventional dairies, reporting that 21.5% of isolates contained gene clusters associated with the production of AMPs (Carson et al., 2017). Several factors could account for this inconsistency, including the use of different databases, which have undergone updates in recent years.

Additionally, variations in methodologies, such as the inclusion of additional steps to assess the completeness of AMP gene clusters utilized in this prior study, may have contributed to these differences. Lastly, it is important to exercise caution when interpreting these results since the mere presence of genes does not necessarily imply their expression or the actual production of AMPs (Sánchez-Romero and Casadesús, 2020).

In the prior chapter (Chapter 5), we found that the presence of "top 10" isolates with MIC against SAU was linked to the presence of IMI against SAU or SSLO, suggesting a connection between *in vitro* antimicrobial activity and the risk of IMI. However, the reasons behind these results remained to be investigated. Thus, for the present study we aimed to explore whether the presence of gene clusters related to the production of AMPs could be linked to the *in vitro* antimicrobial activity against SAU or SSLO IMI, and to determine whether differences in the prevalence of these genes exist between cases and controls. Considering that these AMP gene clusters were in some cases only present in one NASM species, we decided to perform stratified analysis within each of the NASM species. Even with this species-stratified analysis, none of the detected AMP gene clusters were associated with *in vitro* antimicrobial activity. Interestingly, the prevalence of AIPs class I was higher in controls but an opposite result was found for AIPs class II within isolates classified as SCH. Auto-induced peptides are important for SAU pathogenesis, since they play an important role for the induction of expression of VFs through the accessory regulation system (Le and Otto, 2015; Wang and Muir, 2016). In NASM, the production of AIPs can repress the expression of this system in SAU, thus reducing the virulence potential of this pathogen (Canovas et al., 2016; Toledo-Silva et al., 2021). Extensive research has been conducted on this as a potential way of controlling SAU (Gray et al., 2013; Tan et al.,

2018). Therefore, one would expect to see a higher prevalence in controls, as was the case for AIPs class I in SCH, but not for AIPs class II in this microorganism. Additional research is required to gain a deeper understanding of the potential applications of AIPs in the management of mastitis.

6.5.2. Phylogenetic analysis

In this study, we constructed 5 different phylogenetic trees to investigate the evolutionary relationships among NASM isolates of the most common NASM species found in this study, which include SCH, SHAEM, SSC, SSUC, and SXYL/SPXYL. We discovered that different clades within the same species can be recognized, demonstrating the strain diversity within each species and suggesting a complex epidemiology of NASM species. This finding is also consistent with a prior study that showed high strain diversity of NASM (Woudstra et al., 2023). For most species, clade membership was not associated with IMI status, or genotypic or phenotypic *in vitro* antimicrobial activity. An exception to this pattern was SSUC, a bacterium that exhibited the lowest MIC in our study and had the highest proportion of isolates classified within the "top10" group against both SAU and SUB, indicating the highest inhibitory activity within investigated NASM species. In particular, one clade containing 7 genomes showed a lower MIC against both SAU and SUB compared to isolates in other clades. Additionally, all 5 isolates classified as part of the "top 10" group for highest inhibitory activity within SSUC were found in this particular clade, suggesting that something about the genomes of these SSUC isolates may confer protection against SAU and SUB.

Our phylogenetic analysis also revealed that a single cow could harbor multiple strains of a NASM species in a single sample (**Figures 6.2-5;** e.g., cow 171: SHAEM1 and SXYL1, cow

87835: SCH5 and SCH11). This finding indicates that the teat apex can be colonized by multiple strains within the same species. Multi-strain infections have been documented in various human and animal pathogens, including *Staphylococcus* spp. (Balmer and Tanner, 2011). In a few studies, multiple strains of mastitis-causing pathogens have been shown to colonize the mammary gland. For example, prior studies have highlighted this phenomenon in microorganisms such as SAU (Smith et al., 2005) or SUB (Zadoks et al., 2003). However, some evidence suggests that when multiple strains are introduced into the mammary gland, one strain tends to dominate, as shown in a prior study that investigated this phenomenon with SUB (Pryor et al., 2009). The implications of multi-strain infections are still not well understood, but it has been hypothesized that multi-strain infections could have an impact on disease dynamics (Balmer and Tanner, 2011). This may be especially relevant when attempting to draw conclusions about mastitis pathogen epidemiology and for decision making in dairy farms (Exel et al., 2022). Previous studies have indeed documented inter-strain differences in epidemiology (Zadoks et al., 2011), virulence potential (Monistero et al., 2020; França et al., 2021), and antimicrobial activity (Nakatsuji et al., 2017). These findings are also important for study design and research purposes; methodological approaches that do not differentiate at the strain of species levels may yield ambiguous or inaccurate results, and indeed this could explain some of the contradictory or non-concordant results regarding NASM epidemiology (De Buck et al., 2021).

6.5.3. Virulence

In this study, we investigated 207 virulence genes involved in adherence, immune evasion, iron uptake and metabolism or toxin production. These genes were highly prevalent in SAU but showed a lower prevalence in NASM.

Adherence

Adherence-related genes including aap and bap, two genes important for *Staphylococcus* adherence and aggregation into biofilms, showed a higher prevalence than reported in previous studies (Tremblay et al., 2013; Srednik et al., 2017; Naushad et al., 2019; Fergestad et al., 2021). The IcaA gene, vital for adherence and biofilm formation, was present in SCH and SUC isolates in our study, in line with previous reports (Tremblay et al., 2013), although other studies reported a lower prevalence (Srednik et al., 2017; Naushad et al., 2019; Fergestad et al., 2021). Fibronectin binding factors (fnbA and fnbB) are important adhesins that mediate SAU mammary gland invasion (Campos et al., 2022) and were present in nearly all the isolates classified as SAU, as well as those classified as SCH, SXYL and SPSXYL. Lastly, sdr proteins are microbial surface components recognizing adhesive matrix molecules, important for *Staphylococcus* spp. pathogenesis (França et al., 2021), and were present in nearly all isolates.

Exoenzymes

Exoenzymes hold significant importance in the pathogenesis of *Staphylococcus* spp. as they contribute to invasion by breaking down host molecules, thereby enhancing bacterial survival and facilitating dissemination (Tam and Torres, 2019). Nucleases and aur genes were found universally among NASM isolates, consistent with previous research (Naushad et al.,

2019; Fergestad et al., 2021). Other genes exhibited variable prevalence. Adenosine synthase A was exclusive to SCH, SHAEM, and SDEV isolates, lip and geh genes were highly prevalent in SCH, SHAEM, and SXYL, with lower prevalence in SSUC and absence in SSC, all aligning with earlier findings (Naushad et al., 2019).

Immune evasion

All isolates displayed the presence of 1 or more capsular genes, a finding consistent with previous studies (Naushad et al., 2019; Fergestad et al., 2021). This finding highlights the importance of the capsule, as it plays a crucial role in the resistance to phagocytosis (Cheung et al., 2021). Staphylococcal binding immunoglobulin protein and scn are important for SAU immune evasion (Rooijakkers et al., 2005; Smith et al., 2011). In our study, genes encoding these factors were identified in SAU and SCH but not in other NASM species, which agrees with a prior study (Naushad et al., 2019).

Toxins

In our study, enterotoxins were found only in SAU, known for their role in SAU pathogenesis (Cenci-Goga et al., 2003; Pinchuk et al., 2010; de Freitas Guimarães et al., 2013; Patel et al., 2021). Unlike prior studies suggesting enterotoxin production by NASM (Naushad et al., 2019; Fergestad et al., 2021), we did not detect them in NASM, possibly due to species differences. Staphylococcal exotoxins were only identified in SCH. This finding is interesting considering that SCH is the most prevalent NASM isolated from milk samples (De Buck et al., 2021). Many researchers have claimed that SCH has a negative impact on udder health (Supré et al., 2011; Fry et al., 2014; Valckenier et al., 2020), although some other studies have shown a

protective effect of SCH colonization of the mammary gland (De Vliegher et al., 2003), which

suggests the presence of variations in the virulence across different strains. However, genes

encoding staphylococcal exotoxins were found in all SCH isolates, an observation that does not

support the presence of strains with divergent virulence within SCH isolates. Exfoliative toxin

type C was prevalent in almost all NASM isolates, consistent with some studies (Fergestad et al.,

2021), but not with another investigation (Naushad et al., 2019). Beta phenol-soluble modulins

(**PSMs**), important for pathogenesis, were widespread in most NASM except SCH and SSC.

Intriguingly, alpha PSMs, considered highly potent, were absent in both SAU and NASM. Type

VII secretion system proteins are important for virulence of SAU and other gram-positive

pathogens (Spencer and Doran, 2022), and were primarily found in SCH, consistent with

previous studies (Naushad et al., 2019; Fergestad et al., 2021).

6.5.4. Antimicrobial resistance

In this study, we identified that nearly all NASM isolates (97.7%) harbored at least 1

ARG in their genome. In most cases, distribution of ARGs was species dependent; and if a given

ARG was present within a species, it was typically present across all isolates in that species. The

most prevalent ARG was RLMH, a gene that halts action of macrolides and lincosamides, whose

identification was not reported in NASM in a prior study (Nobrega et al., 2018b). In addition, the

prevalence of MPHC, another gene involved in resistance to macrolides, showed a 16.9%

prevalence in our study, which is comparable to that of a prior study (Nobrega et al., 2018b).

Half of the isolates had genes encoding APH3-PRIME, aminoglycoside inactivating enzymes, in

their genomes, which is much higher than reported in a previous study in which the prevalence

of these ARGs was close to zero (Nobrega et al., 2018b). Penicillin resistance in SAU is usually

mediated by the production of beta-lactamases (Nobrega et al., 2018b). In prior studies, SAU and

NASM have shown various degrees of penicillin resistance (Oliver and Murinda, 2012; Nobrega

et al., 2018b; Fergestad et al., 2021). The presence of methicillin resistance in SAU strains,

which have acquired the staphylococcal chromosomal cassette containing mec genes, is a matter

of great concern for both human and animal health (Holmes and Zadoks, 2011; McCarthy et al.,

2012; Paterson et al., 2014; Patel et al., 2021). The prevalence of mec genes was low in our

study, with mecA only identified in SSC. In prior studies, the mecA gene showed a low

prevalence and was previously identified in SSC isolates. However, it was also found in other

species (Nobrega et al., 2018b; Fergestad et al., 2021).

6.5.5. Limitations

One important limitation of our approach is that we focused our investigation on DNA

sequences, while it is known that the presence of a gene does not necessarily guarantee its

expression or the production of a protein (Sánchez-Romero and Casadesús, 2020). Hence, it is

plausible that this could explain some of the inconsistencies between the genotypes (e.g.,

presence of AMP gene clusters) and phenotypes (e.g., *in vitro* inhibitory activity). In addition, it

is conceivable that the AMPs responsible for the observed *in vitro* antimicrobial activity against

SAU or SUB in our study's isolates have not been characterized, are not present in the utilized

database (i.e., Bagel 4), or could not be identified due to bioinformatic challenges (e.g., the lack

of homology in the genes encoding precursor peptides for AMPs) (Morton et al., 2015).

Another limitation of our study was the fact that in the majority of the dairy cows the IMI

was already present on the first postpartum sample after calving. This limits our ability to assess

the temporality between the presence of AMP gene clusters or *in vitro* inhibitory activity and the

onset of new IMI by SAU or SSLO. Lastly, the low sample size (n=80 NASM isolates) and

small number of farms (n=2) limits our ability to make inferences about the different NASM

species, especially those with low prevalence (n<5). This is especially important, considering the

important variation in the distribution of NASM species and strains across different farms.

Therefore, results from this study should only be generalized to the NASM species identified in

the current study and extrapolation to other species should be done with caution.

6.6. CONCLUSIONS

Teat apices of organic dairy cows around parturition harbored more than 1 NASM

species and also in some instances multiple strains within the same species. The prevalence of

AMP associated genes across NASM species was high, with all isolates harboring at least one

AMP associated gene in their genome. The reconstructed phylogenetic tree for each NASM

species contained multiple clades, indicating a non-clonal population that may reflect the

population structure of the cows both within and across the two farms, and/or the evolutionary

history of these bacteria within the two farms. With the exception of SSUC, NASM *in vitro*

antimicrobial activity was not associated with clade membership. Virulence genes were highly

prevalent in SAU, but in most cases showed a low prevalence in NASM species, with a

distribution that appeared to be species-dependent.

6.7. TABLES AND FIGURES

6.7.1. Figures

Figure 6.1. Maximum likelihood phylogenetic tree including 21 isolates identified as *Staphylococcus chromogenes* (SCH). This tree represents a total of 64,794 single nucleotide polymorphisms. Reference: GenBank accession number: GCA_002994305.1. MIC SAU: Minimum inhibitory concentration against *Staphylococcus aureus*. MIC SUB: Minimum inhibitory concentration against *Streptococcus uberis*. Ranking inhibitory activity on SAU: Ranking of isolates according to MIC on SAU. Ranking inhibitory activity on SUB: Ranking of isolates according to MIC on SUB. Auto induced peptide class I (aip_1): NCBI accession number: WP_001093929.1. Auto induced peptide class II (aip_2) NCBI accession number WP_001094921.1. Auto induced peptide class III (aip_3): CBI accession number WP_000735197.1. Auto induced peptide class IV (aip_4) NCBI accession number: WP_001094303.1). Sactipeptides: Interpro accession number PF04055. Subtilosin A NCBI accession number: NP_391616.1. Lanthipeptides class II Interpro accession number: PF05147. Lanthipeptides class IV (Uniprot accession number: O88037). putative bacteriocins 193.2 and 194.2 (putative_bacteriocin_193/194): Genbank accession number AJ002203.2. lacticin Z (lacticin): Interpro accession number PF11758. Carnolysins: Interpo accession number PF00082.

Figure 6.2. Maximum likelihood phylogenetic tree including 19 isolates identified as *Staphylococcus haemolyticus* (SHAEM). This tree represents a total of 52,188 single nucleotide polymorphisms. Reference: GenBank accession number: GCA_001611955.1. MIC SAU: Minimum inhibitory concentration against *Staphylococcus aureus*. MIC SUB: Minimum inhibitory concentration against *Streptococcus uberis*. Ranking inhibitory activity on SAU: Ranking of isolates according to MIC on SAU. Ranking inhibitory activity on SUB: Ranking of isolates according to MIC on SUB. Auto induced peptide class I (aip_1): NCBI accession number: WP_001093929.1. Auto induced peptide class II (aip_2) NCBI accession number WP_001094921.1. Auto induced peptide class III (aip_3): CBI accession number WP_000735197.1. Auto induced peptide class IV (aip_4) NCBI accession number: WP_001094303.1). Sactipeptides: Interpro accession number PF04055. Subtilosin A NCBI accession number: NP_391616.1. Lanthipeptides class II Interpro accession number: PF05147. Lanthipeptides class IV (Uniprot accession number: O88037). putative bacteriocins 193.2 and 194.2 (putative_bacteriocin_193/194): Genbank accession number AJ002203.2. lacticin Z (lacticin): Interpro accession number PF11758. Carnolysins: Interpo accession number PF00082.
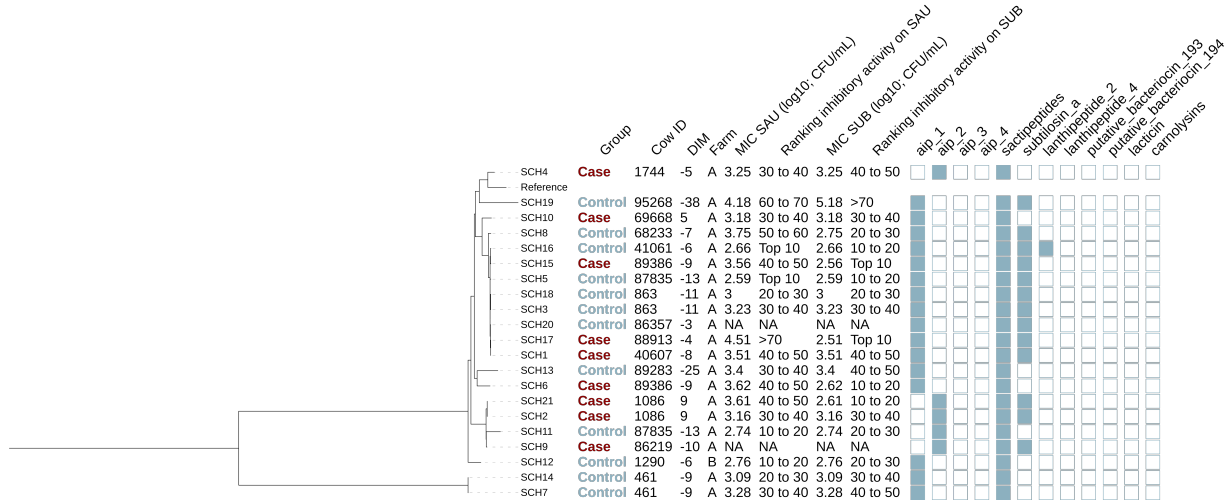
Figure 6.3. Maximum likelihood phylogenetic tree including 6 isolates identified as *Staphylococcus sciuri* (SSC). This tree represents a total of 93,312 single nucleotide polymorphisms. Reference: GenBank accession number: GCA_002209165.2. MIC SAU: Minimum inhibitory concentration against *Staphylococcus aureus*. MIC SUB: Minimum inhibitory concentration against *Streptococcus uberis*. Ranking inhibitory activity on SAU: Ranking of isolates according to MIC on SAU. Ranking inhibitory activity on SUB: Ranking of isolates according to MIC on SUB. Auto induced peptide class I (aip_1): NCBI accession number: WP_001093929.1. Auto induced peptide class II (aip_2) NCBI accession number WP_001094921.1. Auto induced peptide class III (aip_3): CBI accession number WP_000735197.1. Auto induced peptide class IV (aip_4) NCBI accession number: WP_001094303.1). Sactipeptides: Interpro accession number PF04055. Subtilosin A NCBI accession number: NP_391616.1. Lanthipeptides class II Interpro accession number: PF05147. Lanthipeptides class IV (Uniprot accession number: O88037). putative bacteriocins 193.2 and 194.2 (putative_bacteriocin_193/194): Genbank accession number AJ002203.2. lacticin z (lacticin): Interpro accession number PF11758. Carnolysins: Interpo accession number PF00082.
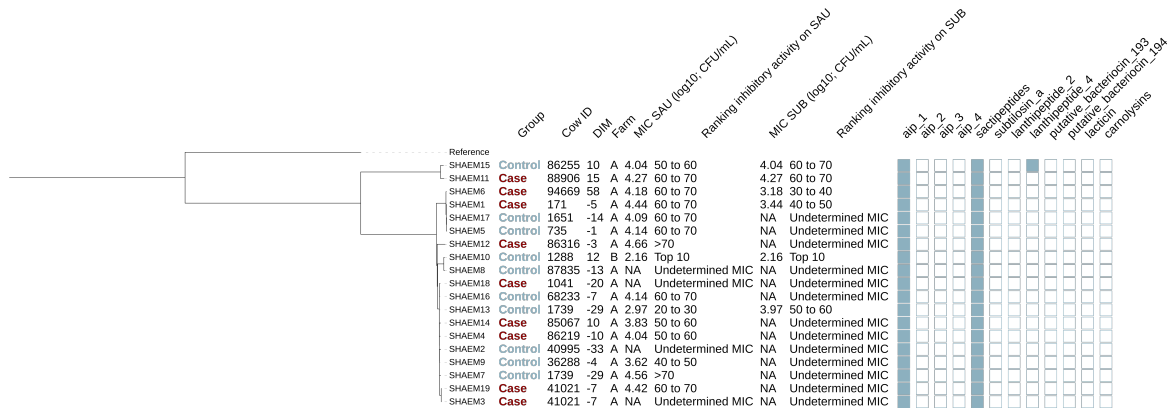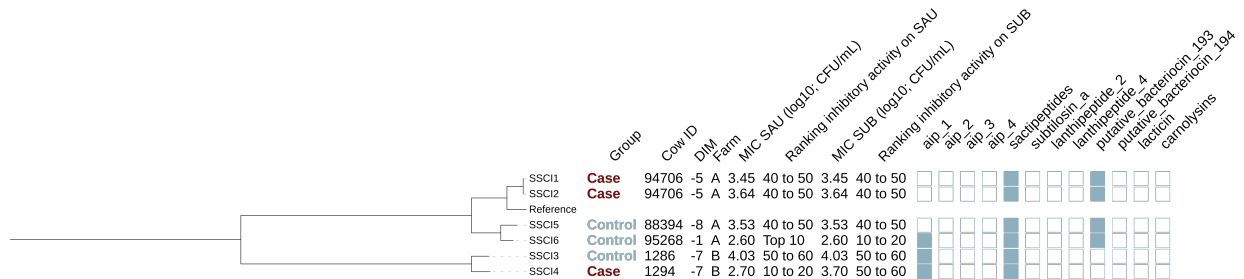
Tree scale: 0.1

	Group	Cow ID	DIM	Farm	MIC SAU (log10; CFU/mL)	Ranking inhibitory activity on SAU	MIC SUB (log10; CFU/mL)	Ranking inhibitory activity on SUB
SSUC11	Case	89386	-9	A	3.68	50 to 60	3.68	50 to 60
Reference								
SSUC8	Case	173	-8	A	3.03	20 to 30	4.03	60 to 70
SSUC6	Control	1333	-5	A	3.11	20 to 30	3.11	30 to 40
SSUC9	Control	86357	-3	A	3.58	40 to 50	2.58	10 to 20
SSUC7	Case	94706	-5	A	4.11	60 to 70	4.11	60 to 70
SSUC12	Case	94706	-5	A	3.14	30 to 40	4.14	60 to 70
SSUC2	Control	95268	-1	A	1.92	Top 10	1.92	Top 10
SSUC10	Control	1333	-5	A	2.65	Top 10	1.65	Top 10
SSUC1	Case	94706	-5	A	2.14	Top 10	2.14	Top 10
SSUC4	Control	95268	-1	A	NA	NA	NA	NA
SSUC3	Case	1041	-20	A	2.28	Top 10	2.28	Top 10
SSUC5	Control	94925	-7	A	2.51	Top 10	2.51	Top 10

Figure 6.4. Maximum likelihood phylogenetic tree including a total of 12 isolates identified as *Staphylococcus succinus* (SSUC). This tree represents a total of 69,716 single nucleotide polymorphisms. Reference: GenBank accession number: GCA_001902315.1. MIC SUB: Minimum inhibitory concentration against *Streptococcus uberis*. Ranking inhibitory activity on SAU: Ranking of isolates according to MIC on SAU. Ranking inhibitory activity on SUB: Ranking of isolates according to MIC on SUB. Auto induced peptide class I (aip_1): NCBI accession number: WP_001093929.1. Auto induced peptide class II (aip_2) NCBI accession number WP_001094921.1. Auto induced peptide class III (aip_3): CBI accession number WP_000735197.1. Auto induced peptide class IV (aip_4) NCBI accession number: WP_001094303.1). Sactipeptides: Interpro accession number PF04055. Subtilosin A NCBI accession number: NP_391616.1. Lanthipeptides class II Interpro accession number: PF05147. Lanthipeptides class IV (Uniprot accession number: O88037). putative bacteriocins 193.2 and 194.2 (putative_bacteriocin_193/194): Genbank accession number AJ002203.2. lacticin z (lacticin): Interpro accession number PF11758. Carnolysins: Interpo accession number PF00082.

Figure 6.5. Maximum likelihood phylogenetic tree including a total of 18 isolates identified as *Staphylococcus xylosus* (XYL) and *Staphylococcus pseudoxylosus* (PXYL). This tree represents a total of 209,336 single nucleotide polymorphisms. Reference: GenBank accession number: GCA_000709415.1. MIC SUB: Minimum inhibitory concentration against *Streptococcus uberis*. Ranking inhibitory activity on SAU: Ranking of isolates according to MIC on SAU. Ranking inhibitory activity on SUB: Ranking of isolates according to MIC on SUB. Auto induced peptide class I (aip_1): NCBI accession number: WP_001093929.1. Auto induced peptide class II (aip_2) NCBI accession number WP_001094921.1. Auto induced peptide class III (aip_3): CBI accession number WP_000735197.1. Auto induced peptide class IV (aip_4) NCBI accession number: WP_001094303.1). Sactipeptides: Interpro accession number PF04055. Subtilosin A NCBI accession number: NP_391616.1. Lanthipeptides class II Interpro accession number: PF05147. Lanthipeptides class IV (Uniprot accession number: O88037). putative bacteriocins 193.2 and 194.2 (putative_bacteriocin_193/194): Genbank accession number AJ002203.2. lacticin z (lacticin): Interpro accession number PF11758. Carnolysins: Interpo accession number PF00082.
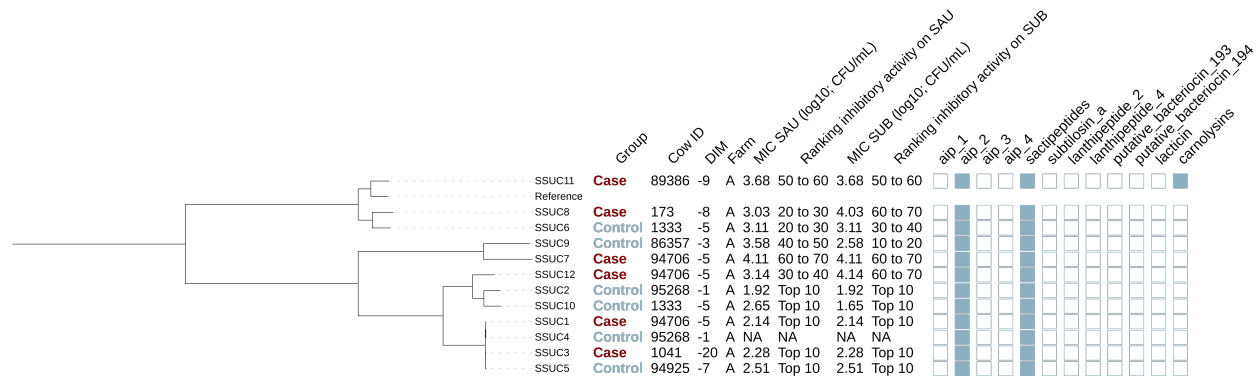
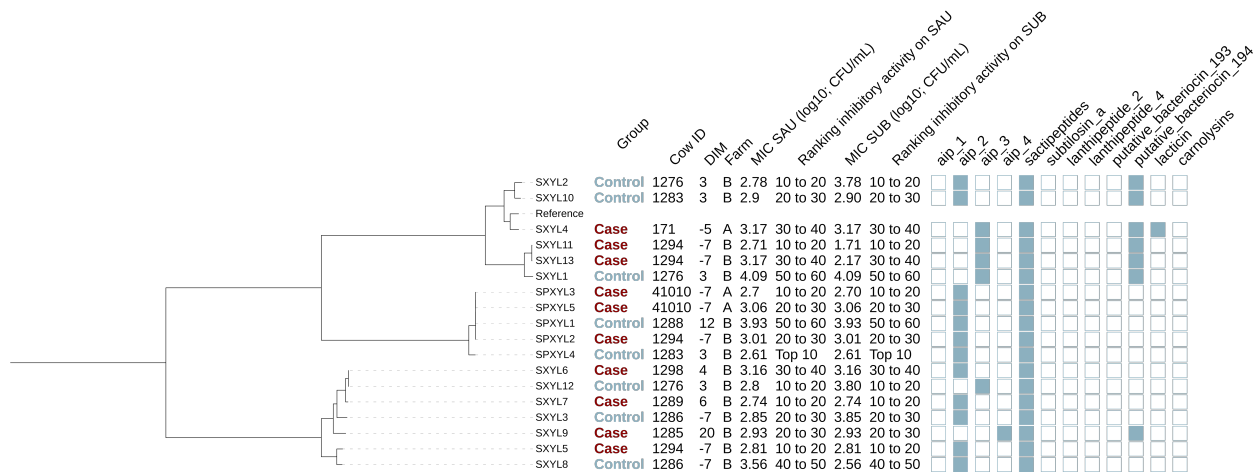**Figure 6.6.** Distribution of virulence genes involved in adherence and exoenzymes in *Staphylococcus aureus* and non-*aureus Staphylococci* and related *Mammaliicoccal* species (NASM). aap: accumulation associated protein. bap: biofilm associated surface protein. atl: autolysin. atlC: fibronectin binding autolysin. clfA: clumping factor class A. clfB: clumping factor class B. cna: collagen adhesin precursor. ebh: cell wall associated fibronectin-binding protein. ebhA: cell wall associated fibronectin-binding protein class A. efb: fibrinogen-binding protein. fnbA: fibronectin-binding protein class A. fnbB: fibronectin-binding protein class B. eap/map: extracellular adherence protein/MHC analogous protein. sasC: cell wall surface anchor family protein type C. sasG: cell wall surface anchor family protein type G. sraP: serin rich adhesion for platelets. icaA: Intercellular adhesion proteins class A. icaB: Intercellular adhesion

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proteins class B. icaC: Intercellular adhesion proteins class C. icaD: Intercellular adhesion proteins class D. IcaR: Intercellular adhesion proteins class R. sdrC: Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein class C. sdrD: Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein class D. sdrE: Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein class E. sdrF: Ser-Asp rich fibrinogen-binding bone sialoprotein-binding protein class F. sdrE: Ger-Asp rich fibrinogen-binding bone sialoprotein-binding protein class G. sdrH: Ger-Asp rich fibrinogen-binding bone sialoprotein-binding protein class H. sdrI: Ger-Asp rich fibrinogen-binding bone sialoprotein-binding protein class I. uafA: cell wall anchored protein class A. adsA: adenosine synthase A. aur: aureolysin. coa: staphylocoagulase. geh: glycerol ester hydrolase. hysA: hyaluronate lyase. lip: triacyglycerol lipase. sak: staphylokinase. nuc: thermonuclease. spLA: serin proteinase class A. splB: serin proteinase class B. splC: serin proteinase class C. splD: serin proteinase class D. splE: serin proteinase class E. splF: serin proteinase class F. sspA: serin protease class A. sspB: serin protease class B. sspC: serin protease class C. vWbp: von Willebrand factor-binding protein.
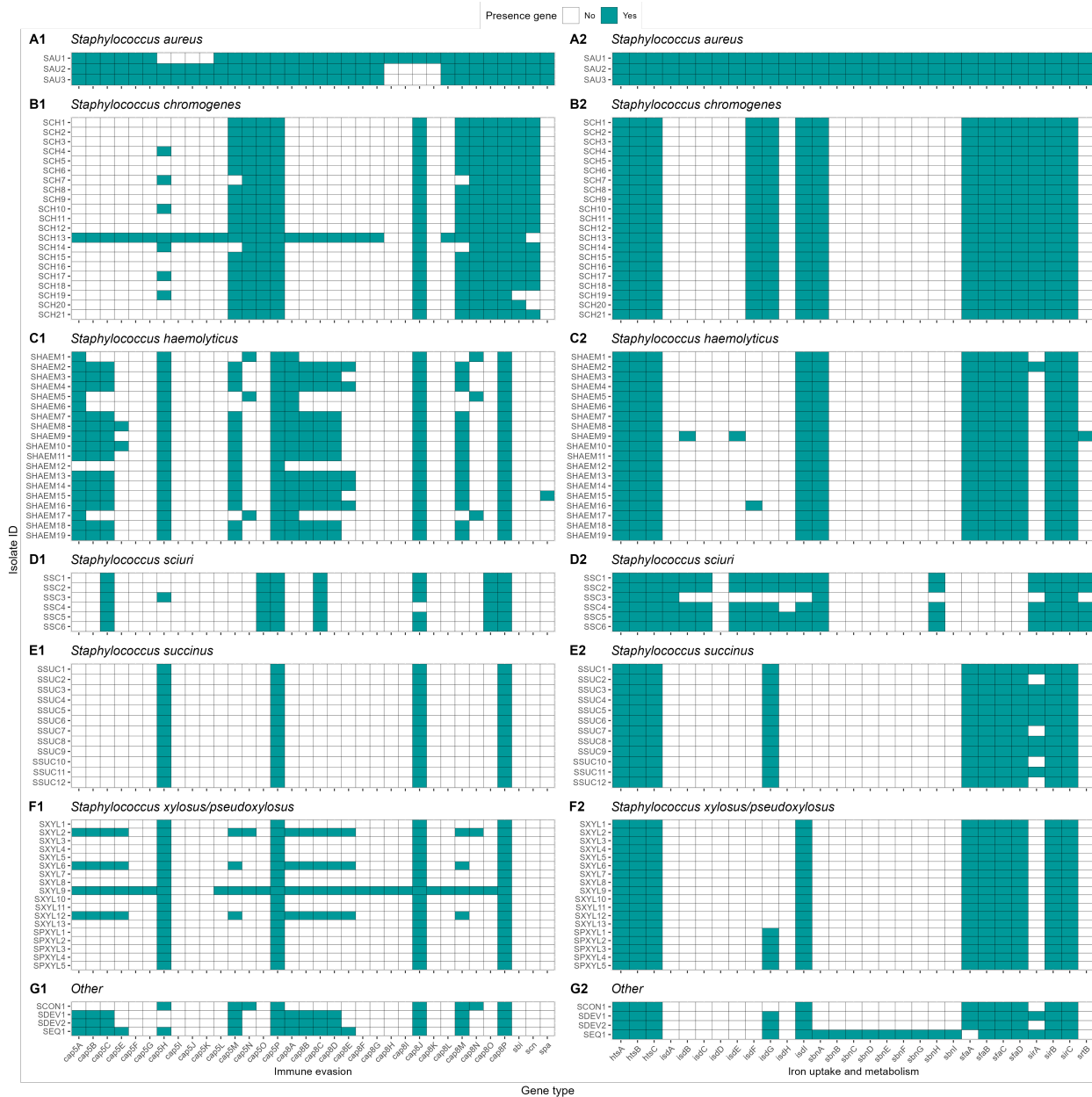
Figure 6.7. Distribution of virulence genes involved in immune evasion and iron uptake and metabolism in *Staphylococcus aureus* and non-*aureus Staphylococci* and related *Mammaliicoccal* species (NASM). cap8A: capsular polysaccharide-synthesis enzyme class 8A. cap8B: capsular polysaccharide-synthesis enzyme class 8B. cap8C capsular polysaccharide-synthesis enzyme class 8C. cap8D: capsular polysaccharide-synthesis enzyme class 8D. cap8E: capsular polysaccharide-synthesis enzyme class 8E. cap8F: capsular polysaccharidesynthesis enzyme class 8F. cap8G: capsular polysaccharide-synthesis enzyme class 8G. cap8H: capsular polysaccharide-synthesis enzyme class 8H. cap8I: capsular polysaccharide-synthesis enzyme class 8I. cap8J: capsular polysaccharide-synthesis enzyme class 8J. cap8K: capsular polysaccharide-synthesis enzyme class 8K. cap8L: capsular polysaccharide-synthesis enzyme

class 8L. cap8M: capsular polysaccharide-synthesis enzyme class 8M. cap8N: capsular polysaccharide-synthesis enzyme class 8N. cap8O: capsular polysaccharide-synthesis enzyme class O. cap5A: capsular polysaccharide-biosynthesis protein class 5A. cap5B: capsular polysaccharide-biosynthesis protein class 5B. cap5C: capsular polysaccharide-biosynthesis protein class 5D. cap5E: capsular polysaccharide-biosynthesis protein class 5E. cap5F: capsular polysaccharide-biosynthesis protein class 5F. cap5H: capsular polysaccharide-biosynthesis protein class 5H. cap5I: capsular polysaccharide-biosynthesis protein class 5I. cap5J: capsular polysaccharide-biosynthesis protein class 5J. cap5L: capsular polysaccharide-biosynthesis protein class 5L. cap5M: capsular polysaccharide-biosynthesis protein class 5M. cap5N: capsular polysaccharide-biosynthesis protein class 5N. cap5O: capsular polysaccharide-biosynthesis protein class 5O. cap5P: capsular polysaccharide-biosynthesis protein class 5P. chp: chemotaxis-inhibiting protein. sbi: staphylococcal binding immunoglobulin protein. scn: staphylococcal complement inhibitor. spa: staphylococcal protein A. isdaA: iron-regulated surface determinant protein class A. isdaB: iron-regulated surface determinant protein class B. isdaC: iron-regulated surface determinant protein class C. isdaD: iron-regulated surface determinant protein class D. isdaE: iron-regulated surface determinant protein class E. isdaF: iron-regulated surface determinant protein class F. isdaG: iron-regulated surface determinant protein class G. isdaH: iron-regulated surface determinant protein class H. isdaI: iron-regulated surface determinant protein class I. htsA: FecCD iron compound ABC transporter permease family protein class A. htsB: FecCD iron compound ABC transporter permease family protein class B. htsC: FecCD iron compound ABC transporter permease family protein class C. sbnA: siderophore biosynthesis class A. sbnB: siderophore biosynthesis class B. sbnC: siderophore biosynthesis class C. sbnD: siderophore biosynthesis class C. sbnE: siderophore biosynthesis class E. sbnF: siderophore biosynthesis class F. sbnG: siderophore biosynthesis class G. sbnH: siderophore biosynthesis class H. sbnI: siderophore biosynthesis class I. srtB: sortase B. sirA: staphylococcal iron regulated class A. sirB: staphylococcal iron regulated class B. sirC: staphylococcal iron regulated class C. sfaA: staphyloferrin A biosynthesis protein class A. sfaB: staphyloferrin A biosynthesis protein class B. sfaC: staphyloferrin A biosynthesis protein class C. sfaD: staphyloferrin A biosynthesis protein class D.
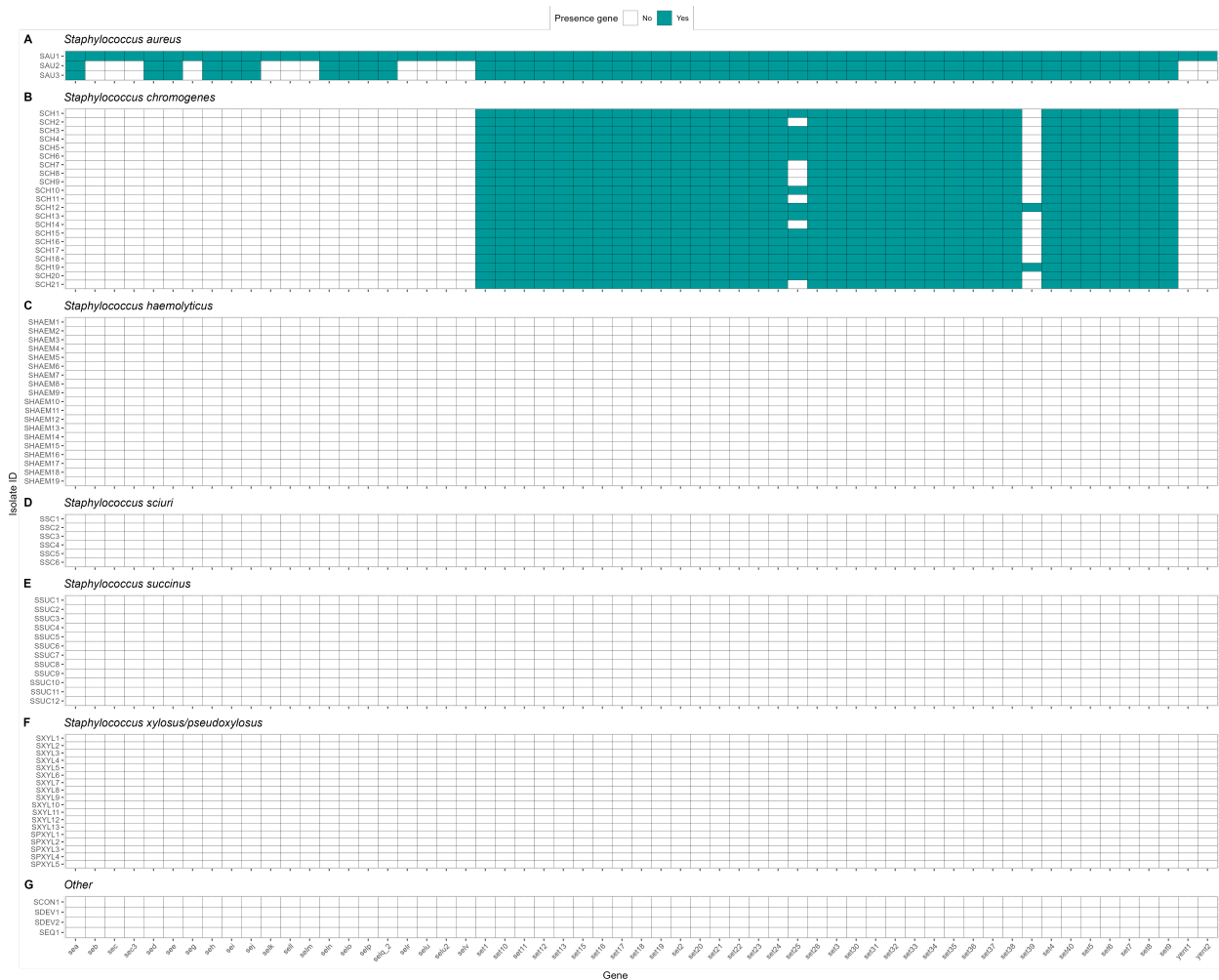
Figure 6.8. Distribution of enterotoxins and Staphylococcal exotoxin genes in *Staphylococcus aureus* and non-*aureus Staphylococci* and related *Mammaliicoccal* species (NASM). sea: Staphylococcal enterotoxin A. seb: Staphylococcal enterotoxin B. sec: Staphylococcal enterotoxin C. sec3: Staphylococcal enterotoxin C1 precursor. sed: Staphylococcal enterotoxin D. see: Staphylococcal enterotoxin E. sef: Staphylococcal enterotoxin F. seg: Staphylococcal enterotoxin G. seh: Staphylococcal enterotoxin H. sei: Staphylococcal enterotoxin I. sej: Staphylococcal enterotoxin J. selk: Staphylococcal enterotoxin K. sell: Staphylococcal enterotoxin L. selm: Staphylococcal enterotoxin M. seln: Staphylococcal enterotoxin N. selm: Staphylococcal enterotoxin M. seln: Staphylococcal enterotoxin N. selo: Staphylococcal enterotoxin O. selp: Staphylococcal enterotoxin P. selq: Staphylococcal enterotoxin Q. selr: Staphylococcal enterotoxin R. selu: Staphylococcal enterotoxin U. selu2: Staphylococcal enterotoxin U2. selv: Staphylococcal enterotoxin v. set1: staphylococcal exotoxin 1. set2: staphylococcal exotoxin 2. set3: staphylococcal exotoxin 3. set4: staphylococcal exotoxin 4. set5: staphylococcal exotoxin 5. set6: staphylococcal exotoxin 6. set7: staphylococcal exotoxin 7. set8: staphylococcal exotoxin 8. set9: staphylococcal exotoxin 9. set10: staphylococcal exotoxin 10. set11: staphylococcal exotoxin 11. set12: staphylococcal exotoxin 12. set13: staphylococcal

exotoxin 13. set14: staphylococcal exotoxin 14. set15: staphylococcal exotoxin 15. set16: staphylococcal exotoxin 16. set17: staphylococcal exotoxin 17. set18: staphylococcal exotoxin 18. set19: staphylococcal exotoxin 19. set20: staphylococcal exotoxin 20. set21: staphylococcal exotoxin 21. set22: staphylococcal exotoxin 22. set23: staphylococcal exotoxin 23. set24: staphylococcal exotoxin 24. set25: staphylococcal exotoxin 25. set26: staphylococcal exotoxin 26. set27: staphylococcal exotoxin 27. set28: staphylococcal exotoxin 28. set29: staphylococcal exotoxin 29. set30: staphylococcal exotoxin 30. set31: staphylococcal exotoxin 31. set32: staphylococcal exotoxin 32. set33: staphylococcal exotoxin 33. set34: staphylococcal exotoxin 34. set35: staphylococcal exotoxin 35. set36: staphylococcal exotoxin 36. set37: staphylococcal exotoxin 37. set38: staphylococcal exotoxin 38. set39: staphylococcal exotoxin 39. set40: staphylococcal exotoxin 40. yent1: enterotoxin yent1. yent2: enterotoxin yent2.
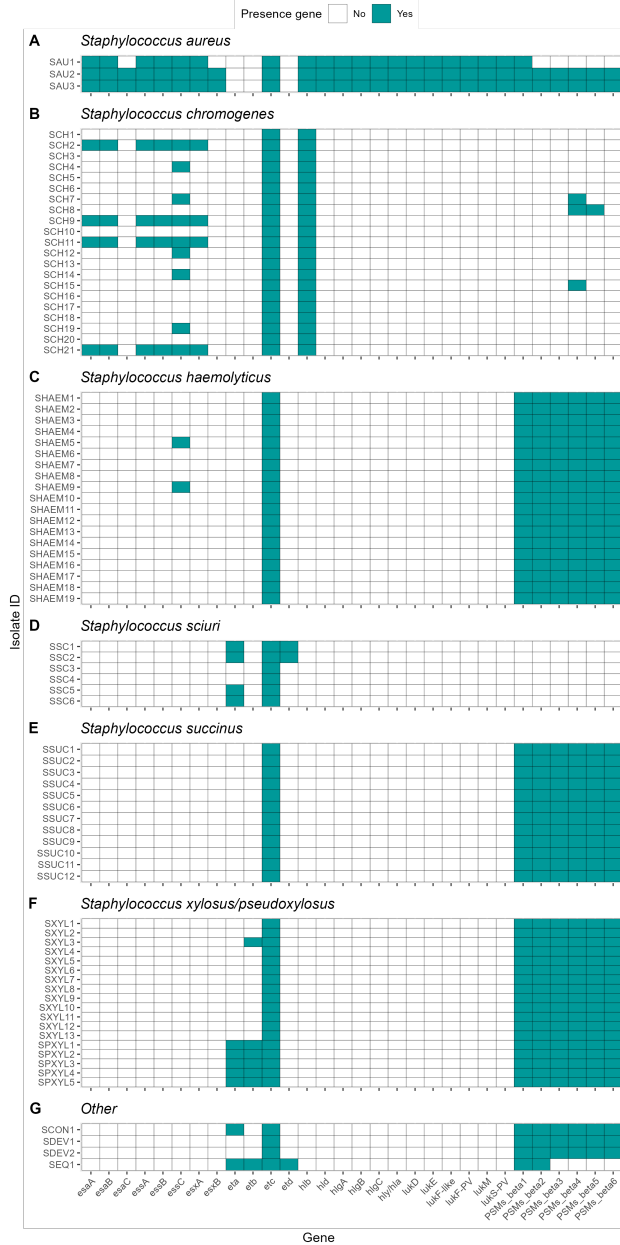
Figure 6.9. Distribution of various toxin genes *Staphylococcus aureus* and non-*aureus Staphylococci* and related *Mammaliicoccal* species (NASM). hly/hla: alpha-hemolysin. Hlb: beta-hemolysin. hld: delta-hemolysin. hlgA: gamma-hemolysin component A. hlgB: gamma-hemolysin component B. hlgC: gamma-hemolysin component C. lukM: leukocidin M. lukF-like: Panton-Valentine leukocidin LukF-PV chain precursor. lukF-PV: Panton-Valentine leukocidin chain F precursor. lukS-PV: Panton-Valentine leukocidin chain S precursor. lukE: leukotoxin E. lukD: leukotoxin D. tsst: toxic shock syndrome toxin-1. eta: exfoliative toxin class A. etb: exfoliative toxin class B. etc: exfoliative toxin class C. etd: exfoliative toxin class D. esaA: type VII secretion system protein class A. esaB: type VII secretion system protein class B. esaC: type VII secretion system protein class C. essA: type VII secretion system protein class A -

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monotopic membrane protein. essB: type VII secretion system protein class B - monotopic membrane protein. essC: type VII secretion system protein class C - FtsK/SpoIIIE family ATPase. esxA: type VII secretion system protein secreted protein class A. esxB: type VII secretion system protein secreted protein class B. PSMs_beta1: Phenol-soluble modulin beta 1. PSMs_beta2: Phenol-soluble modulin beta 2. PSMs_beta3: Phenol-soluble modulin beta 3. PSMs_beta4: Phenol-soluble modulin beta 4. PSMs_beta5: Phenol-soluble modulin beta 5. PSMs_beta6: Phenol-soluble modulin beta 6.
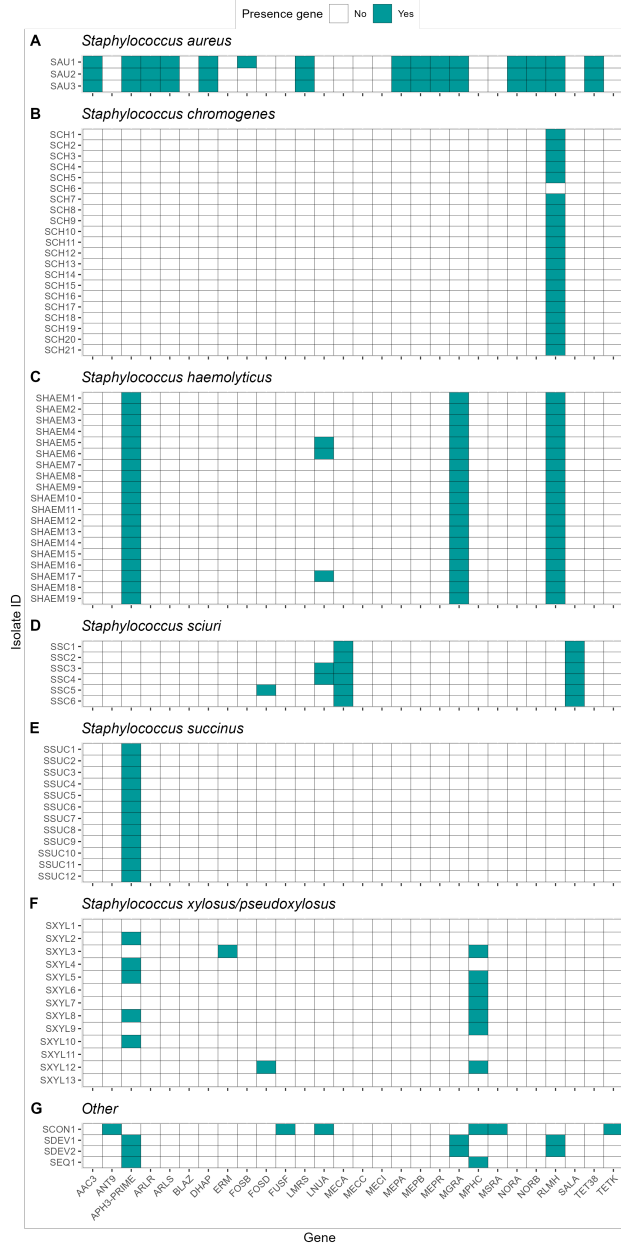
Figure 6.10. Distribution of antimicrobial resistance genes in *Staphylococcus aureus* and non-*aureus Staphylococci* and related *Mammaliicoccal* species (NASM). AAC3: Aminoglycoside N-acetyltransferase. ANT9: Aminoglycoside nucleotidyltransferase. APH3-PRIME: Aminoglycoside O-phosphotransferases. ArlR is a response regulator that binds to the norA promoter to activate expression. ArlR must first be phosphorylated by ArlS. ArlS : protein histidine kinase that phosphorylates ArlR, a promoter for norA expression. BLAZ: Class A betalactamases. DHAP: Phenicol_resistance_MFS_efflux_pumps. ERM: 23S rRNA methyltransferases. FOSB: Fosfomycin thiol transferases class B. FOSD: Fosfomycin_thiol_transferases class D. FUSF: Fusidic_acid_esterases. LMRS: Drug and biocide MFS efflux pumps. LNUA: Lincosamide_nucleotidyltransferases. MECA: Penicillin binding

protein class A. MECC: Penicillin binding protein class C. MECI: Penicillin binding protein class I. MEPA: Drug and biocide MATE efflux pumps class A. MEPB: Drug and biocide MATE efflux pumps class B. MEPR: Drug and biocide MATE efflux pumps class R. MGRA: MDR regulator. MPHC: Macrolide phosphotransferases. MSRA: MLS resistance ABC efflux pumps. NORA: Drug and biocide MFS efflux pumps class A. NORB: Drug and biocide MFS efflux pumps class B. RLMH: 23S rRNA methyltransferases. SALA: Multi-drug ABC efflux pumps. TET38: Tetracycline resistance MFS efflux pumps. TETK: Tetracycline resistance MFS efflux pumps.

CHAPTER 7: CONCLUSIONS

Previous studies have suggested that udder health may be impaired in organic dairy farms. However, there is limited knowledge about the udder health of dairy cows on organic dairy farms. Especially, prior research mostly addressed herd-level management, leaving gaps in our understanding of individual cow-level mastitis pathogen prevalence on organic dairy farms. Given the potential impact of antibiotic restrictions on organic dairies, further research is needed to understand how mastitis pathogens persist in the udder and their association with udder health and milk production.

Commensal microorganisms, particularly non-*aureus Staphylococci* and closely related *Mammaliicoccal* species (**NASM**), have gained attention as potential candidates for controlling mastitis. Multiple studies have demonstrated their *in vitro* antimicrobial activity against mastitis pathogens. Nevertheless, some questions require further research: Does this *in vitro* antimicrobial activity protect against colonization by major pathogens? What drives the antimicrobial activity of NASM? Lastly, is this effect strain-specific and associated with specific clades within each NASM species? To address these questions, the main objectives of this dissertation were to: 1) To describe the intramammary infection (**IMI**) dynamics of primiparous cows on certified organic farms during early lactation; 2) To investigate the relationship between presence and persistence of IMI during the first 35 days in milk (**DIM**), and somatic cell count (**SCC**) and milk production up to 180 DIM; 3) To explore the *in vitro* antimicrobial activity of NASM from the teat apices of organic cows with and without IMIs caused by *Staphylococcus aureus* (**SAU**), or *Streptococcus* spp. and *Streptococcus*-like organisms (**SSLO**); 4) To investigate the genotype of NASM isolates from the teat apices of dairy cows with and without an IMIs caused by *Staphylococcus aureus*, or SSLO.

Given the limited knowledge regarding the prevalence and distribution of mastitis pathogens on organic dairy farms, Chapter 3 investigated the dynamics of IMI in a longitudinal study that involved 503 first-lactation organic dairy cows from five farms, with weekly sampling conducted during the first five weeks after calving. This study provided an overview of the prevalence and persistence of IMI in early lactation first-lactation dairy cows on organic dairy farms, revealing a high prevalence of SAU, NASM and SSLO in the enrolled animals during the start of their first lactation, with differences in prevalence observed among herds. *Staphylococcus aureus*, *Staphylococcus chromogenes*, and *Streptococcus* spp., showed high prevalence at calving and persisted in the mammary gland, whereas NASM non-*chromogenes*, SLO, and gram-negative microorganisms primarily caused transient IMI. Collectively, our results emphasize the importance of focusing on prepartum management for IMI prevention and control in organically reared first-lactation cows.

Considering the restrictions placed on antibiotics use on organic dairy farms, it is possible that this could alter the association between IMI and lactational SCC and milk production. The fourth chapter investigated the association between presence and persistence of IMI in the first 35 DIM and high SCC (defined as SCC >200,000 cells/mL) as well as milk production up to 180 DIM. The presence of *Staphylococcus* spp. and *Streptococcus* spp. IMI within the first 35 DIM was associated with an increased risk of high SCC, whereas gram-negative bacteria or *Streptococcus*-like organisms showed no association with this outcome. Milk production in one or more postpartum monthly tests was reduced in cows with *Streptococcus* spp. and NASM IMI in the first 35 DIM. *Bacillus* spp. IMI in early lactation was associated with a lower risk of high SCC and higher milk production. The relationship between IMI and high SCC was more

pronounced for persistent IMI but not evident for transient IMI. Our results advance our understanding of the association between IMI in early lactation, lactational SCC and milk production in first-lactation organic dairy cows.

While some studies have shown evidence of antimicrobial activity of NASM, it is unclear whether this could lead to protection against the risk of IMI by major mastitis pathogens. To address this knowledge gap, in the fifth chapter, we conducted a case control study, to explore the *in vitro* antimicrobial activity of NASM isolated from the teat apex of organic cows against SAU and *Streptococcus uberis* (**SUB**). The NASM isolates used in this study were isolated one week before the diagnosis (or absence) of an IMI caused by SAU or SSLO. In this investigation, the presence of NASM isolates classified as the "top 10" with the lowest MIC against SAU, based on the ranking of all isolated from both our case and control cows, was highest in cows without SAU or SSLO IMI. These results provide initial information about the inhibitory capabilities of naturally occurring NASM against mastitis pathogens (namely SAU and SUB), contributing to our understanding about the relationship between the commensal NASM population in the teat skin and udder health in dairy cows.

In the sixth chapter, we investigated the phylogeny of NASM isolates whose *in vitro* inhibitory activity was examined in the preceding chapter. Our goal was to analyze their gene distribution in relation to antimicrobial peptides, virulence factors, antimicrobial resistance, and their associations with *in vitro* inhibitory activity. Our results showed that all isolates had at least one gene associated with the production of antimicrobial peptides in their genome. Presence of genes related to the production of antimicrobial peptides showed no association with their *in vitro* inhibitory capacities or the presence of major mastitis pathogens IMI. Among NASM

species, only *Staphylococcus succinus* showed an association between clade membership and *in vitro* inhibitory activity. Specifically, within this microorganism, one clade of isolates exhibited strong *in vitro* inhibitory activity against both SAU and SUB. Virulence genes were highly prevalent in SAU. However, in most cases, they exhibited a low prevalence in NASM species, with a distribution that appeared to be species-dependent. This study deepens our knowledge about the genomes of NASM collected from the teat apices of organic dairy cows and their potential antimicrobial activity against major mastitis pathogens.

As a whole, the research described in this dissertation allows us to better understand the epidemiology of mastitis causing pathogens in organically managed first-lactation cows, and the relationship between IMI by different microorganisms on udder health and milk production. Our investigation also provides evidence suggesting that commensal NASM from the teat apex offer a defense against the invasion of mammary gland pathogens. Our investigation was unable to identify potential genetic determinants of the *in vitro* inhibitory activity of NASM against mastitis pathogens. Despite all the findings described in this thesis, more research is needed to determine potential mechanisms behind the inhibitory effects of NASM on mastitis pathogens and identify the specific antimicrobial peptides responsible for these effects.

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